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CONDUCTIVITY AND PERMEABILITY.

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, July 19, 1921.)

When an electrical current passes from a salt solution into a living cell, ions must enter the protoplasm. An increase in the permeability of the protoplasm to ions must decrease its electrical resistance, and *vice versa*. The electrical resistance of the protoplasm may therefore be regarded as a measure of its permeability to ions.

If we attempt to measure the electrical resistance of the protoplasm we must first consider the structure of the tissue. For example, we find in the case of *Laminaria* that the protoplasm of each cell forms a thin layer which surrounds a large central vacuole filled with cell sap. Since experiments have shown that the cell sap has about the same electrical resistance as the solution which bathes the cell, it is evident that when the electrical resistance of the cell increases, on transferring it from sea water to another solution of the same conductivity, the change must be due to an increase in the resistance of the thin layer of protoplasm which bounds the cell. This has led the writer to assume that the resistance is proportional to a substance, M, at the surface of the cell; if M forms a layer at the surface it is obvious that an increase in the thickness of this layer will increase the resistance, and *vice versa*. It is therefore assumed that the resistance depends upon the amount of M which is present in the surface.[1]

In *Laminaria* the protoplasmic masses (cells) are separated from each other by a thin layer of gelatinous substance (cell wall). In passing through the tissue a part of the current goes through the protoplasm and another part passes between the protoplasmic masses,

[1] This assumption is simple and facilitates quantitative treatment. It is recognized that changes in resistance might depend upon other properties of this layer, and that the layer need not necessarily be continuous.

in the substance of the cell wall.[2] Consequently when we employ the electrical method we must ascertain whether we are investigating the permeability of the protoplasm or merely that of the cell wall.

Obviously the best method of attacking this problem is to kill the tissue by such means (*e.g.*, partial drying, heating to 35°C., weak alcohol, etc.) as can not alter the cell wall, and then investigate its behavior under the influence of various reagents. We find that all of these methods produce the same result. After death the tissue no longer shows the changes in resistance which are observed when living tissue is subjected to the influence of reagents. It is therefore evident that the changes are due to the living protoplasm.

The cell wall appears in all cases to have practically the same conductivity as the surrounding solution. If we subject living tissue to solutions of the same conductivity, but of different chemical composition, the resistance of the cell wall remains unaltered while that of the protoplasm undergoes great variations. If, for example, living tissue is placed in a solution of NaCl or $CaCl_2$ (of the same conductivity as sea water) its behavior differs. In NaCl the resistance falls; in $CaCl_2$ it rapidly rises and later falls to a minimum. We infer that the permeability of the protoplasm increases in NaCl; and that in $CaCl_2$ there is a decrease followed by an increase.

This is in complete agreement with results obtained when permeability is measured by such methods as plasmolysis,[3] specific gravity,[4] tissue tension, exosmosis, and diffusion through living tissue.[5] This agreement indicates that the electrical method measures the permeability of the protoplasm. It is however desirable to go further, if possible, and analyze the factors involved in electrical resistance.

[2] As explained in a former paper (Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxvi, 485) the fact that a part of the current passes through the protoplasm is shown by the fact that $CaCl_2$ raises the resistance of living tissue and by the fact that the temperature coefficient of electrical conductivity differs in living and dead tissue.

[3] Osterhout, W. J. V., *Science*, N. S., 1911, xxxiv, 187.

[4] Loeb, J., *Science*, N. S., 1912, xxxvi, 637. *Biochem. Z.*, 1912, xvii, 127.

[5] Brooks, S. C., *Proc. Nat. Acad. Sc.*, 1916, ii, 569. For exosmosis of the pigment of *Rhodymenia* in relation to electrical resistance see Osterhout, W. J. V., *J. Gen. Physiol.*, 1919, i, 299.

If we consider the behavior of the current from this point of view, it is evident that in the simplest cases, where the plant is a membrane only one cell thick (as in *Porphyra* and *Monostroma*) and the current passes through this membrane at right angles to its surface, we need consider only a single cell and its adjacent cell wall, as shown in Fig. 1, *A*. The part of the current which goes through the protoplasm may be designated as C_P. while that which traverses the cell may be called C_W.

Experiments show that the resistance of the living tissue is much greater than that of tissue which has been carefully killed with all possible precautions to prevent any alteration of the cell wall.[6] We therefore feel confident that the conductivity of the living protoplasm is less than that of the cell wall.

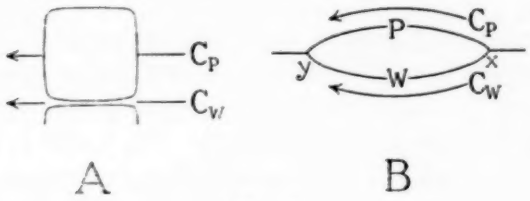

FIG. 1.

In order to see how the current may distribute itself let us suppose the protoplasm to be replaced by a wire,[7] *P*, as in Fig. 1, *B* and the cell wall to be replaced by a wire, *W*. The current flowing between

[6] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1919, i, 299. *J. Biol. Chem.*, 1918, xxxvi, 485.

[7] We might consider the protoplasm to be replaced by two wires one of which corresponds to the thin layers of protoplasm which are traversed by the current in a direction at right angles to their planes, the other corresponding to the similar layers of protoplasm in each cell (around the edges of the cell shown in Fig. 1, A) in which the current flows in the plane of the layer. It is evident, however, that these latter may be neglected in our calculations since they occupy such exceedingly small fractions of the cross-section.

If we neglect these we may say that in traversing a cell the current passes through a thin layer of cell wall and then one of protoplasm (in both cases at right angles to the plane of the layer), then through the cell sap, and finally through a layer of cell wall and one of protoplasm (at right angles to their planes). It is evident that in this case we may neglect the effect of the cell wall and of the cell sap since their resistance is very small in comparison with

the points X and Y in the wire P may be called C_P; that in the other wire C_W. The total current, C, flowing between X and Y will be the sum of the partial currents, or,

$$C = C_P + C_W$$

We may consider the current (conductance) as equal to the reciprocal of the resistance and write

$$\frac{1}{R} = \frac{1}{R_P} + \frac{1}{R_W}$$

in which R is the total resistance between X and Y, R_P is the resistance of the wire P, and R_W, that of W. Applying this equation to *Laminaria*[8] (and expressing the resistance in the usual way as the per cent of the normal) we may calculate the values of C_W, C_P, R_W, and R_P.

Under normal conditions in sea water, the resistance is taken as 100 and therefore $C = 1 \div 100$ but in certain solutions (having the same conductivity as sea water) the resistance may rise to 300 or more; and in this case C would equal $1 \div 300 = .0033$ (or less), and since some of it must flow in the protoplasm the amount which traverses the cell wall must be less than this. We are therefore safe in putting it as low as $1 \div 350 = .002857$.

All the experiments hitherto made indicate that the conductivity of cell the wall remains unaltered in spite of changes in the chemical

that of the protoplasm and is in series with it. We may therefore consider the protoplasm to be replaced by a single wire having a resistance equal to that of the two layers of protoplasm which are traversed by the current in a direction at right angles to their planes.

[8] So far we have considered only the simplest case, when the plant is only one cell thick. But it is evident that these considerations also apply when several membranes are placed together, forming a mass comparable to the tissue of *Laminaria*. The only difference is in that case the current would traverse a very thin layer of cell wall in passing from one protoplasmic mass to the next, so that what we have spoken of as the resistance of the protoplasm would be composed in part of the resistance of these cell walls. When the protoplasm is dead the total resistance is only 10.29 and the resistance of these cell walls must be only a small fraction of this. Consequently their resistance in the living tissue of *Laminaria* is undoubtedly less than 1 when that of the protoplasm is 140. The resistance of these cell walls may therefore be neglected.

character of the solution, provided the conductivity of the solution remains the same. We may therefore take .002857 as the fixed value of C_W.

Let us now consider what values C_P assumes as the resistance changes. In sea water we have[9] R = 100 and

$$C = \frac{1}{100} = .002857 + C_P$$

whence $C_P = .007143$ and $R_P = 1 \div C_P = 140$. In the same manner we find that when $R = 90$, $R_P = 121.15$, and when $R = 10$, $R_P = 10.29$.

The changes in resistance thus far discussed have been treated as though they occurred in sea water; in this case the experiments indicate that the conductivity of the cell sap remains practically constant and hence need not be taken into account in our calculations. We may now ask whether this is also the case when the changes in resistance occur in other solutions. In order to investigate this, experiments were made with solutions of NaCl and $CaCl_2$ (of the same conductivity as sea water). The tissue was placed in these solutions and removed after various intervals of exposure. It was cut into small bits and ground (so as to open the cells) and the conductivity of the expressed juice was compared with that of sea water. As no significant difference was found we may consider that the conductivity of the cell sap does not change sufficiently in these solutions to alter our calculations.

Let us now consider the changes in protoplasmic resistance which occur in toxic solutions. When tissue is placed in NaCl 0.52 M the net resistance falls rapidly. The death curve may be obtained by means of the formula[10]

$$\text{Resistance} = 2700\left(\frac{K_A}{K_M - K_A}\right)\left(e^{-K_A T} - e^{-K_M T}\right) + 90e^{-K_M T} + 10$$

[9] The total conductance of the protoplasm is greater than that of the cell walls, but the protoplasm occupies a much greater fraction of the conducting cross-section than the cell walls, so that the actual conductivity of the protoplasm is much less than that of the cell wall.

[10] For the explanation of this formula see Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533; *J. Gen. Physiol.* 1920–21, iii, 145, 415, 611.

in which T is the time of exposure, K_A and K_M are constants, and e is the basis of natural logarithms. We find by means of this formula that in a solution[11] of NaCl 0.52 M (for which $K_A = .018$ and $K_M = .540$) the net resistance after 10 minutes is 87.76 per cent of the normal; after 30 minutes it is 64.26, and after 60 minutes it is 41.62. Knowing the net resistance we can calculate the protoplasmic resistance, as explained above. After 10 minutes the protoplasmic resistance is 117.12 per cent (corresponding to the net resistance of 87.76 per cent). Since it is desirable to express all resistances as per cent of the resistance in sea water we divide 117.12 by 140 (which is the protoplasmic resistance in sea water) and obtain 83.66 per cent. Proceeding in this way we find that after 30 minutes the

TABLE I.

Velocity Constants at 15°C.

$CaCl_2$ in solution.	$CaCl_2$ in surface.	K_A	K_M	K_{AP}	K_{MP}
per cent	*per cent*				
0	0	0.018	0.540	0.0234	0.702
1.41	12.5	0.000222	0.00666	0.000293	0.00878
2.44	20.0	0.000187	0.00546	0.000237	0.00708
4.76	33.33	0.000245	0.00590	0.00032	0.007136
15.0	63.73	0.000364	0.0073	0.0005035	0.00855
35.0	84.34	0.000481	0.00859	0.000678	0.00955
62.0	94.22	0.00053	0.009	0.000761	0.00989
100.0	100.0	0.0018	0.0295	0.002685	0.0323

protoplasmic resistance is 56.22 per cent and after 60 minutes 33.74 per cent. In order to fit the formula to these values we must change the constants, putting $K_{AP} = 0.0234$ (in place of $K_A = 0.018$) and $K_{MP} = 0.702$ (in place of $K_M = 0.54$). It is therefore evident that in changing from net resistance to protoplasmic resistance we merely shift the value of the constants. The question arises whether this affects the general conclusions drawn from the study of net resistance. In order to decide this question the constants for $CaCl_2$ and

[11] See Osterhout, W. J. V., *J. Biol. Chem.*, 1917, xxxi, 585.

for various mixtures of NaCl and $CaCl_2$ were ascertained; these are given in Table I.[12]

There are two points of principal importance in the consideration of these constants: (1) It was shown in a former paper[13] (which dealt with net resistance only) that the value of $K_A \div K_M$ increases regularly as the per cent of $CaCl_2$ in the surface of the cell increases. That this is also true in the case of protoplasmic resistance is evident from Fig. 2. (2) It was also pointed out that as the per cent of $CaCl_2$ in the solution decreases from 62 to 1.41 per cent the value of K_M first decreases (reaching a minimum at 4.76 per cent) and then increases. It was found that the amount of decrease corresponds to

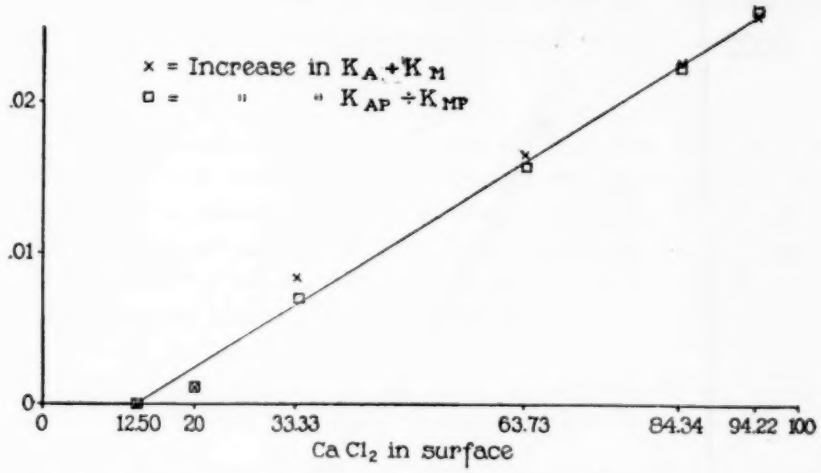

FIG. 2. Ordinates represent the increase in value of $K_A \div K_M$ and of $K_{AP} \div K_{MP}$ In each case the value given represents the increase over the corresponding value in the solution containing 1.41 per cent $CaCl_2$ (the corresponding per cent in the surface being 12.5). Abscissæ represent per cent of $CaCl_2$ in the surface. In order to facilitate comparison the values of $K_{AP} \div K_{MP}$ have been divided by 1.685.

[12] These are approximate values, obtained graphically. The constants of the curves of protoplasmic resistance are designated as K_{AP} (corresponding to K_A) and K_{MP} (corresponding to K_M). The curves of protoplasmic resistance may show less inhibition at the start than those of net resistance.

[13] Osterhout, W. J. V., *J. Gen. Physiol.*, 1920–21, iii, 415.

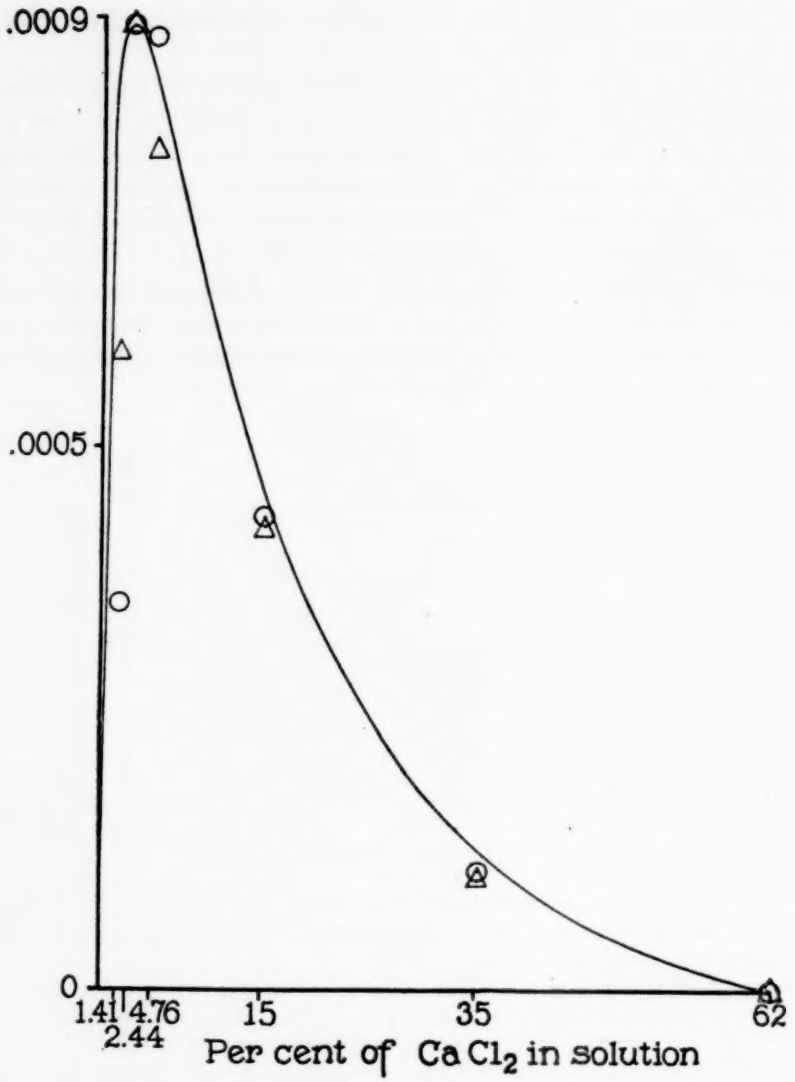

FIG. 3. Ordinates represent the amount of Na_4XCa and also the decrease in the value of K_M(Δ) and of K_{MP}(o) as compared with the corresponding value in the solution containing 62 per cent $CaCl_2$. Abscissæ represent per cent of $CaCl_2$ in the solution. In order to facilitate comparison the values of K_M have been multiplied by 0.251 and those of K_{MP} by 0.321.

the amount of a hypothetical salt compound (Na_4XCa). This is also true in the case of protoplasmic resistance, as shown in Fig. 3.[14]

It would therefore appear that we arrive at the same conclusions whether we study net resistance or protoplasmic resistance. When the solution is changed the constants change in a corresponding manner in both cases, the only difference being in their absolute values, but it is evident that in this case differences in absolute values are of no importance.

It should be emphasized that this general conclusion would remain valid in case it should be found that the values given in this paper for C_P and C_W are incorrect. There seems to be no doubt that the value of C_W is constant under the conditions of these experiments and as long as this is true the conclusions drawn from the study of net resistance apply also to protoplasmic resistance.

SUMMARY.

An electrical current passing through a living plant flows partly through the cell wall and partly through the protoplasm. The relative amounts of these two portions of the current can be calculated.

The outcome of such calculations shows that the conclusions drawn from the study of the resistance of the tissue as a whole apply also to the resistance of the protoplasm, and consequently to the permeability of the protoplasm to ions.

[14] A rough calculation shows that this is also true of K_{NP} and K_{OP} (corresponding to the K_N and K_O mentioned in the former paper[13]).

STEREOTROPIC REACTIONS OF THE SHOVEL-NOSED RAY, RHINOBATUS PRODUCTUS.

By S. S. MAXWELL.

(From the Rudolph Spreckels Physiological Laboratory of the University of California, and the Scripps Institution for Biological Research.)

(Received for publication, June 14, 1921.)

I.

It has been pointed out by Loeb[1] that our orientation in space is determined mainly by three groups of tropistic influences; namely, light, gravitation, and contact. Light and gravitation cause the orientation of organisms through effects upon muscle tonus. When the lines of force strike the animal obliquely, as for example, when light rays fall unequally on the two eyes, the unequal stimulation causes differences of tonus on the two sides, and the symmetrically placed muscle groups acting with unequal strength, bring about forced changes in the direction of locomotion. When the lines of force coincide with the axis of symmetry, or the plane of symmetry of the body, the effects are equal on the two sides and movement can go forward in a straight line. For the contact, or stereotropic, reactions, quantitative relations of this nature have not heretofore been described.

In my studies on the physiology of the labyrinth I have found it necessary to distinguish carefully between those eye and fin movements which result from excitations of end-organs in the ear, and movements which arise from other sources. In this way I have come to make observations on the contact reactions of the shovel-nosed ray, or guitar fish, *Rhinobatus productus*, which will, I believe, throw important light on the nature of stereotropic reactions in general.

Rhinobatus is not so broadly expanded as most of the other rays. The pectoral fins, however, have the characteristic fleshy thickened

[1] Loeb, J., Forced movements, tropisms, and animal conduct, Philadelphia and London, 1918.

base. The posterior part of the body and the tail are shark-like in appearance. The eyes are freely movable and can be elevated or retracted in a manner quite similar to the eye movements of the frog. Specimens 3 to 4 feet in length may be taken, but the reactions about to be described are better seen in the smaller animals, 15 to 18 inches long.

When this animal is placed on a shark board and supplied with plenty of aerated sea water through a rubber tube, little or no tying is necessary to keep it in position. Under these conditions a contact stimulus applied to the upper surface of the head or snout excites certain very definite coordinated movements of the fins and eyes, the particular combination of movements depending on the locus and strength of the stimulus.

II.

If the skin of a *Rhinobatus* is gently stroked with the finger or with a blunt instrument at any point along the midline of the head, for example, between *7* and *8* (Fig. 1), both eyes are retracted, the movements of the two being approximately equal. If a similar stimulus is applied near the outer margin of the upper surface of the head, as at *1* (Fig. 1), the eye on that side is retracted strongly, the other eye is moved very little or not at all. If trials are made at other places, *e.g.*, at *2* or *3*, Fig. 1, it is seen that as the point stimulated approaches the midline the amount of movement of the two eyes becomes more and more nearly equal, or in other words, the relative amount of retraction of each eye varies inversely with its distance from the point of application of the stimulus.

It was relatively easy to record these movements graphically. An Engelmann pincette was attached to each eye by a fold of the integument just where the rudimentary lid passes over into the cornea. The pincettes were connected by threads to a pair of light heart levers in such a way that retraction of an eye gave an upward direction to the curve. In the tracing here reproduced, Fig. 2, the upper lever was connected with the left eye and the lower with the right. The writing points were placed as nearly as possible in the same vertical line but in order to make the relations more certain simultaneous ordinates were marked throughout. The small rhythmi-

cal oscillations are respiratory, rather than eye movements. Spontaneous, "voluntary" movements occur occasionally, as between *6* and *7* near the end of the tracing. In this experiment the stimulus

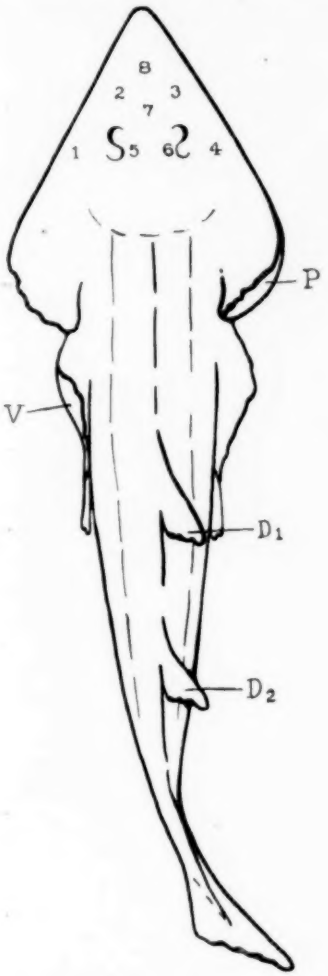

FIG. 1. Diagram of the dorsal view of *Rhinobatus productus*.

employed was a gentle stroke with the finger. These strokes were made as nearly equal as possible, but the method could hardly be expected to give perfectly uniform results. The numbers at the bottom of the tracing show the points stimulated as charted on Fig. 1.

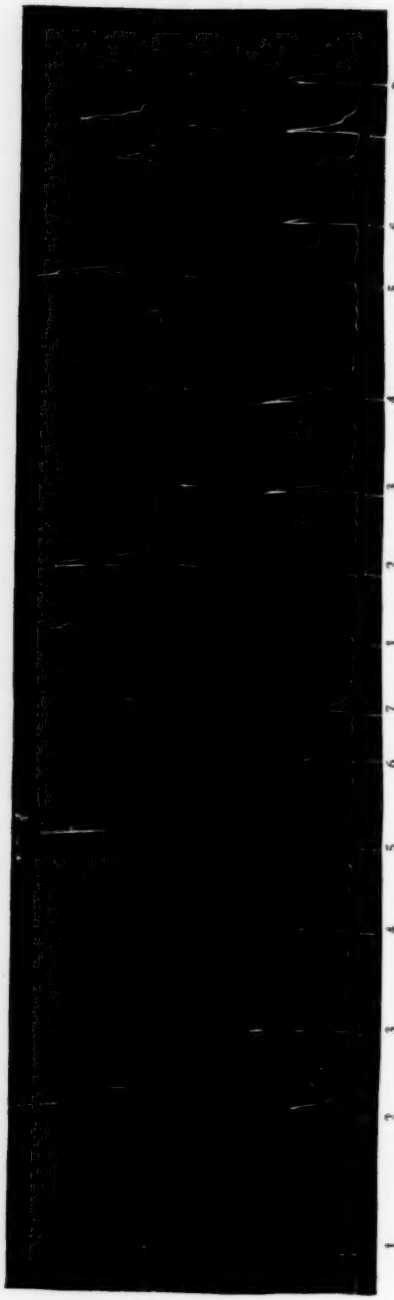

FIG. 2. Upper curve shows movements of left eye; lower curve, right eye. The numbers show the points stimulated, as indicated on Fig. 1.

Certain peculiarities remain to be mentioned. While the responses could be obtained from contact stimuli on all parts of the upper surface of the head, some parts were noticeably more sensitive than others. Also some parts were less likely than others to produce the bilateral response. Thus the strength of stimulus used in securing the tracing reproduced, rarely gave rise to a retraction of both eyes when applied at *5* or *6*, very near the inner margin of the eye. Stimuli applied to the lower surface of the snout, even near the lateral margin where the upper surface was very sensitive, were very slightly or not at all effective.

The movements which I have just described are retraction of the bulbs and partial closure of the rudimentary lids, and are not at all to be confused with the conjugate movements which result from excitation of the labyrinth.

The contact stimuli which elicit the eye movements in *Rhinobatus* bring about at the same time a remarkable group of coordinated movements of the fins and tail. An asymmetrically applied stimulus, *e. g.*, at *1* or *2* (Fig. 1), on the left side of the head, causes elevation of the posterolateral margin of the right pectoral, P, and of the left pelvic fin, V, while both dorsal fins, D_1 and D_2, are flexed to the right. A slightly stronger stimulus causes, in addition, a bending of the tail to the right and a slight elevation of the anterolateral margin of the left pectoral fin. If the stimulus is applied to the right side all the relations are, of course, reversed; the left pectoral and right pelvic fins are elevated and the dorsal fins and tail are turned to the left. If the animal was moving forward in the water the effect of the new positions of the fins would be to alter the direction so as to terminate the contact with the stimulating object. If for example the point touched was at *2*, Fig. 1, on the left upper surface of the head, the left side of the head and body would be lowered and at the same time the animal would veer off to the right; in other words a definite, negatively stereotropic, reaction would result. The fin and eye movements are as clear and characteristic in their way as are those which result from stimulation of the labyrinth.

In the preceding paragraph I have described the effect of a moderate stimulus. If a more severe stimulus is applied, whether to the midline or to a point asymmetrically situated on the upper

surface of the head, a different reaction results; the margins of all four of the paired fins are elevated strongly. The effect of this would be to check the forward movement in the water and at the same time to steer the fish downward to the bottom. This is plainly also an example of a negatively stereotropic reaction. (It is necessary to bear in mind that a stimulus on the upper midline of the head is symmetrically placed with reference to the median plane of the animal but not with reference to the horizontal plane.)

All of the above reactions occur with great regularity and may be called forth over and over again by appropriate stimuli, but it is important to remember that the character of the response depends not only on the location but also on the nature of the stimulus. A stimulus at a given point may cause a change of direction of locomotion in the horizontal plane with a slight rotation of the body around its longitudinal axis, while a stronger stimulus at the same point may cause a change of direction out of the horizontal plane, that is, a movement toward the bottom.

III.

The above described reactions occur equally well in animals in which the forebrain has been destroyed. I have made repeatedly transsections of the brain as far back as the optic chiasma without affecting them in the least. Complete destruction of the two labyrinths is equally without effect. Since these movements occur in the absence of the forebrain it would be illogical to speak of them as "voluntary," or "purposeful," or "instinctive." On the other hand they illustrate beautifully the tropistic conception of animal behavior since they are very evidently reactions of the organism as a whole in response to asymmetrically applied stimuli. The effect of these stimuli is to bring about sudden changes of tonus in those groups of muscles which in their state of resting equilibrium hold the eyes and fins in a position of symmetry. The change of tonus causes an unsymmetrical action of the corresponding muscle groups on the two sides of the body with the result that the new position induced, terminates the contact with the stimulating object.

At first sight it might appear that these reactions differ in their nature from the other tropisms because in the latter we are concerned

with the influence of forces acting along definite lines, while in the contact reaction the stimulus is applied to a single spot or a limited area of the skin. It is hardly necessary to point out that in heliotropic animals with two eyes the light rays act upon two very limited areas, namely, portions of the retinas, and in the geotropic reactions, gravitation acts upon very limited areas in the internal ear to bring about or maintain orientation. While ordinarily the two eyes or the two ears come into play in the heliotropic and geotropic reactions respectively, experiments show that marked effects are produced by stimuli applied to one eye or one ear alone. Another apparent difference is that contact stimuli may act from moment to moment in different directions. This, however, would be paralled by the effect of an intermittent, moving light upon a heliotropic organism.

It is of interest to picture the behavior of the organism under the play of two tropistic influences. Instead of a direct response to either, the position or movement which occurs may be the simple resultant of the two, as in the case of barnacle larvæ exposed to two lights from different directions.[2] On the other hand the one stimulus suddenly applied may for the moment inhibit the effect of the other. The free swimming fish, for example, reacts to gravitation by definite compensatory movements and positions of the eyes and fins through which it maintains a definite course and a horizontal position. If now suddenly a foreign body comes in contact with a certain portion of the head, say a point on the left upper surface, a negatively stereotropic movement occurs; the fins are thrown into an unsymmetrical position causing the left side of the head to be lowered and the course to be changed to the right. These changes terminate the contact and the stereotropic reaction ceases. But the sudden swing to the right has excited the ampulla of the right horizontal canal and a compensatory movement to the left, *i.e.*, to the original course, is produced while at the same time the rotation around the longitudinal, body axis has stimulated the otolith organs and the ampullæ of the vertical canals in such way that the horizontal position is again attained. The resulting behavior of the animal would in this way

[2] Loeb, J., and Northrop, J. H., Heliotropic animals as photometers on the basis of the validity of the Bunsen-Roscoe law for heliotropic reactions, *Proc. Nat. Acad. Sc.*, 1917, iii, 539.

give the appearance of volition and purpose, where a more exact analysis shows its purely mechanical nature. In case more than two sets of influences come into play simultaneously the behavior becomes more variable, the analysis becomes more difficult, and the behavior gives the appearance of purpose or caprice, although its correct interpretation would involve no new factors such as "will" or "intelligence," but merely the recognition of a larger number of variables.

THE STEREOTROPISM OF THE DOGFISH (MUSTELUS CALIFORNICUS) AND ITS REVERSAL THROUGH CHANGE OF INTENSITY OF THE STIMULUS.*

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In the preceding paper[1] I have described certain contact reactions of an elasmobranch fish, *Rhinobatus productus*, and I have shown that these reactions are definitely tropistic in their nature. I pointed out that in *Rhinobatus* the response to a particular stimulus depends upon two factors (*a*) the strength and (*b*) the location of the stimulus; that, for example, a weak stimulus applied to the right upper surface of the head causes the fins to assume an asymmetrical position of such character that the body momentarily swerves to the left, and at the same time the right side of the head is depressed; but a strong stimulus applied at the same point brings about such a change in the position of the fins as would arrest the forward movement of the animal and cause it to dive to the bottom. The effect of both these modes of reaction is to terminate the contact with the stimulating object and hence they are both to be regarded as examples of negative stereotropism.

When I attempted to find out whether analogous reactions could be obtained from other elasmobranchs I was at first greatly puzzled by the behavior of the common dogfish. A dogfish tied down on the shark board and supplied with a current of aerated sea water would respond to stroking or scratching stimuli applied to the head or snout with decided movements or changes or position of the fins; but the results were often confusing or contradictory. A contact

*The expense of this research has been met in part by an appropriation from the Board of Research of the University of California.

[1] Maxwell, S. S., Stereotropic reactions of the shovel-nosed ray (*Rhinobatus productus*), *J. Gen. Physiol.*, 1921–22, iv, 11.

stimulus applied to the right upper surface of the snout would at one moment cause the dorsal fins to turn to the right, while at another moment a stimulation of the same region caused these fins to bend to the left. The paired fins and the tail participated in these responses, and the direction of their movements had a definite relation to the movements of the dorsal fins. It became apparent that these fin movements were always consistent among themselves; they were more than simple reflexes, and showed a coordinated adjustment of the organism as a whole. In general they could be seen to exhibit such an arrangement as would be necessary to turn the animal either in the direction of the stimulating object or away from it. That is to say, the reactions were in each case stereotropic, but the sense of the stereotropism could be positive or negative. It became then a matter of interest to determine, if possible, the conditions of the reversal, and so to control these conditions as to make the responses predictable. This proved to be indeed very simple.

METHODS.

In making these experiments on the effects of contact stimuli it would have been desirable to keep the fish in its natural position in the water. This however was impracticable because the mechanical effect of the stroke or push which constitutes the stimulus was sufficient to move the body of the fish under the unstable conditions of water support only. Moreover the stimulus excited movements of locomotion and the observer was unable to keep track of the positions and changes of positions of the different fins. If the aquarium used was large the fish was soon out of reach; if small, new stimuli were offered by collision with the walls. Another disturbing factor, if the animal is floating in the water and free to move, is the fact that each response to a tactile stimulus causes such a change of position as to excite the labyrinth and thus introduce other reflexes. It was necessary, therefore, to use the ordinary method of artificial respiration by means of a current of aerated sea water through a rubber tube in the animal's mouth.

When the dogfish is first placed in the shark board rather violent struggles occur, and tying is usually necessary until the animal be-

comes quiet. After a few minutes of immobility the cords can be gently loosened and removed and the experiment can go on for some time without any need of artificial restraint. This is important because experiments on contact stimuli should not be complicated by possible inhibitions or reenforcements from the presence of the binding cords. It is true that the ventral surface of the body is still in contact with the board, but this is not an unnatural situation since the animal when free often rests for long periods on the bottom of the aquarium. In order better to observe the movements of the paired fins the animal was usually placed above the board on a thick piece of wood no wider than the body, thus allowing the pectorals to project like wings.

The reactions about to be described were obtained by stroking or scratching the outer margin of the head from near the snout to a point just below the eye. It was not necessary that the stroke be carried the whole distance; a short stroke or sometimes a mere touch anywhere within the region mentioned gave the same result. It is not to be inferred that analogous reactions are not elicited by contact stimuli applied to other regions. I have confined this paper to reactions from the parts mentioned for the sake of definiteness of description and interpretation.

Strength of Stimulus and Sense of Reaction.

For most dogfish a stroke with a finger wet with sea water was sufficient to produce a definite response. As a more severe stimulus I used a scratch with the points of a small pair of forceps. The first of these usually corresponds to the designation "weak" the other "strong" stimulus.

It soon became apparent that fairly constant responses could be obtained if the stimuli were of uniform intensity. In fact under favorable conditions the movements could be repeated over and over with machine-like regularity. The following portion of the record of an experiment is typical (Table I). The pauses between the successive trials were merely the time necessary to set down the results.

Weak Stimuli.—Inspection of the results of the above experiment shows that when a weak stimulus is used the dorsal fins and the

tail turn toward the stimulated side. The effect of these as a steering apparatus would be to change the course toward the stimulated side; *e.g.*, turning the dorsal fins or the tail to the left would cause the course to swerve to the left. But in addition to this another effect

TABLE I.

Mustelus californicus, 33 Inches Long, May 20, 1921.

Stimulus.		Reaction.				
Kind.	Side.	D1.	D2.	Tail.	Right Pectoral.	Left Pectoral.
Weak (Finger).	Left.	Left.	Left.	Left.	Down ╱	Up ╲
	Right.	Right.	Right.	0	Up ╲	Down ╱
	Left.	Left.	Left.	Left.	Down ╱	Up ╲
	Right.	Right.	Right.	Right.	?	Down ╱
	Left.	Left.	Left.	Left.	Down ╱	Up ?
	Right.	Right.	Right.	?	Up ╲	Down ╱
Strong (Forceps).	Left.	Right.	Right.	Right.	Down ╲	Up ╱
	Right.	Left.	Left.	Left.	Up ╱	Down ╲
	Left.	Right.	Right.	?	Down ╲	Up ╱
	Right.	Left.	Left.	Left.	Up ╱	Down ╲
Weak (Finger).	Left.	Left.	Left.	Left.	Down ╱	Up ╲
	Right.	Right.	Right.	Left?	Up ╲	Down ╱
	Left.	Left.	Left.	0	Down ╱	Up ╲
	Right.	Right.	Right.	Right.	Up ╲	Down ╱
Strong (Forceps).	Left.	Right.	Right.	Right.	Down ╲	Up ╱
Repeated many times over with like results.						

The first column indicates the strength of stimulus; the second, the side of the head to which it is applied; the third, fourth, and fifth, the direction of movement of the first and second dorsal and the tail fins respectively. The last two columns give the direction of movement of the anterior border of the right and left pectoral fins; and, in these two columns, ╱ at the end of the word indicates that the posterior end of the fin was higher than the anterior; ╲, that the posterior margin was lower than the anterior.

would result. When a dorsal fin turns to the left it assumes an oblique position; that is, it is its posterior border which goes to the left most strongly. Its resistance as the animal moves forward in the water would have a screw effect, tending to rotate the body around its longitudinal axis so that the ventral side would be turned in the di-

rection of the stimulating object. This rotation effect would be increased by the new position of the pectoral fins. The pectoral on the stimulated side is elevated but its posterior margin is raised less than its anterior or is even depressed; the pectoral of the other side makes a movement which is just the converse. These fins would then also havea screw effect tending to the same direction of rotation as the dorsals, namely, ventral side toward the stimulating object. The reaction is clearly tropic and in the positive sense.

It will be seen that the total effect of a weak stimulus is to turn the ventral side of the animal, as well as to swerve the course, in the direction of the stimulating object. This accords well with what one sees on watching the dogfish swimming about in a small aquarium. They are often seen going round and round, keeping near the walls, with the body tilted to one side so that the mouth and belly are turned somewhat toward the wall. This is just the position which would be produced by the above reactions, if, on making the turn at a corner, the edge of the snout came slightly in contact with the wall. Sometimes I have been able to see such contacts actually occurring, but the asymmetrical position was often assumed when the wall was not touched. In this case it might be that the increased pressure or resistance of the water when the fish was moving near the wall could act as a stimulus. Indeed I found that a spurt of water from a pipette could be used instead of a finger stroke as a weak stimulus.

Since in the dogfish the mouth is far back on the ventral surface of the head it is not unreasonable to suppose that the positive stereotropic reaction assists in the capture of food; the response to a contact stimulus would tend at once to bring the mouth into position to seize the stimulating object.

Strong Stimuli.—The experiments described above show that the reaction to a strong stimulus is almost exactly the reverse of the reaction to a weak stimulus. The dorsal fins and the tail are flexed to the side away from the contact. The pectoral fin on the stimulated side is elevated, its posterior margin still more than its anterior, the pectoral on the opposite side is depressed, the posterior margin more than the anterior. The whole arrangement of the fins is that of a screw whose effect in the water would be to rotate the body around its longitudinal axis in such a way as to turn the back to the stimulat-

ing object. At the same time the dorsal fins and the tail would act as a steering apparatus to alter the course to a direction away from the source of stimulation. This then is also a definite tropic reaction and in the negative sense.

Attention should perhaps be called to the fact that while the positive reaction is on the whole opposite in character to the negative it is not precisely so. A weak stimulus on the right side of the snout or a strong stimulus on the left side would each tend to turn the ventral side to the right and cause the course to veer to the right. But the mechanism is not quite the same so far as the pectorals are concerned. In the positive reaction the pectorals act feebly in comparison to the unpaired fins; in the negative reaction their movement is relatively more vigorous. In both the positive and the negative reaction the pectoral of the stimulated side is elevated; but in the one case its posterior margin is elevated less than the anterior and in the other case more. It will not do then to say that the negative reaction differs from the positive merely in the fact that the excitation is shunted from one side of the central nervous system to the other. It is certainly not so simply diagrammatic as that.

The Decerebrate Animal.

My experiments on *Rhinobatus* led me to expect that the destruction of the forebrain would have no effect on the character of the stereotropic reactions of the dogfish. In a number of instances I made transections of the brain, usually near the anterior margin of the cerebellum, with no noticeable alteration in the responses to contact stimuli. The following record of an experiment will serve as an example:

July 4, 1921.—Mustelus californicus, 29 inches long.

9:20 a.m. Brain exposed and cut across at anterior margin of cerebellum. Animal returned to tank, lies inert; does not right itself.

10:30 a.m. Animal swimming about normally. Taken out and placed on board. Contact reactions tested. (Table II.)

Possible Sources of Error.

Certain possible sources of error were considered and should be mentioned.

1. Reflexes from the Labyrinth.—I have already spoken of the necessity of avoiding any movement of the head at the moment of the experiment. It would be quite possible, if labyrinth effects were not taken into account, that the mechanical effect of the stroke used as the stimulus would turn the head enough to excite a reflex from the internal ear. I have often in the course of an experiment tried to see how much and how fast the head must be turned in order to provoke a labyrinthine response, and the amount has always been much greater than could be caused by the strongest contact stimulus employed. But in order to avoid all possibility of error from this source I have made experiments on a number of dogfish in which both labyrinths

TABLE II.

Stimulus.		Reaction.				
Kind.	Side.	D1.	D2.	Tail.	Right Pectoral.	Left Pectoral.
Forceps scratch.	Right.	Left.	Left.	Left.	Up.	Down.
	Left.	Right.	Right.	Right.	Down.	Up.
	Right.	Left.	Left.	Left.	Up.	Down.
	Left.	Right.	Right.	Right.	Down.	Up.
Finger stroke.	Right.	Right.	Right.	0	Up.	Down.
	Left.	Left.	Left.	Left.	Down.	Up.
	Right.	Right.	Right.	Right.	Up.	
	Left.	Left.	Left.	Left.	Down.	Up.
	Continues to respond like a normal animal.					

had been previously destroyed and have found the stereotropic responses in no way altered.

2. Tension of Neck and Trunk Muscles.—The observation of Lyon[2] that eye movements can be elicited by bending the body of the dogfish, even after total destruction of the ears, can be easily repeated. Fin movements can also be obtained in the same way. It was conceivable, then, that the responses or some of them might have been due to pressure on the side of the head inducing reflexes by changes of tension in the joints of the neck region. It was easy to test this also

[2] Lyon, E. P., Compensatory motions in fishes, *Am. J. Physiol.*, 1900–01, iv, 77.

and I found that the amount of bending necessary to produce any reflex movement in this way was vastly more than could be caused by the contact stimuli.

3. Retinal Stimuli.—I have already mentioned the fact that dogfish swimming about in the concrete tank often keep close to the wall, with the dorsal fins slightly flexed and the body tilted so that the belly is turned slightly outward toward the wall. It was possible that this position was induced by the moving image of the wall upon the retina. In making the strokes used as contact stimuli I naturally passed my hand close to the eye of the stimulated side. It was possible that the flexion of the dorsal fins might be due in reality to the image (or the shadow) of the hand upon the retina. I found in fact that when the fish was placed on the board parallel to the window, so that the left eye was toward the light, passing the hand between the window and the animal's left eye often caused a definite flexing of the first dorsal fin to the left, but I could never by this means get a movement of the other dorsal or of the paired fins. In order, however, to test this matter farther I made many experiments in which the eyes were covered with thick pads of wet absorbent cotton and found no apparent change in the responses to contact stimuli.

CONCLUSIONS.

In the majority of instances the regularity of the responses to the finger strokes and to the forceps scratches is no more remarkable than the definiteness of the change from the one kind of response to the other. The two kinds of stimuli mentioned differed sufficiently to give reactions of opposite sign. An occasional animal, however, reacted very feebly or not at all to the finger stroke, and gave "positive" reactions to fairly strong forceps scratches, in fact in a few instances no negative reaction was obtained. Other specimens gave positive reactions to moderately strong forceps scratches (stimuli which in the great majority of specimens would cause a lively negative reaction), but gave the negative reaction when still more force was applied. On the other hand a few gave only the negative reaction to any effective stimulus.

It was noticeable, too, that what constituted a "weak" or a "strong" stimulus depended upon the physiological state of the individ-

ual animal. Specimens were usually less sensitive and less responsive immediately after the struggles connected with capture and immobilization were over than they were ten or fifteen minutes later. In fact an occasional animal remained unresponsive until roused to excitability by an unusually hard scratch or a pinch or twist of the tail, when it suddenly began to react in the regular way. These instances forcibly reminded one of the awakening from a nap. Immediately after the "awakening" all the responses, even to strong stimuli, were likely to be positive, although occasionally just the reverse was the case. Then after a few strokes the reactions would become normal, that is, positive to weak and negative to strong stimuli.

Naturally one raises the question: How is the reversal brought about? The phenomena described in this paper seem to present a close analogy to the observation of Sherrington[3] that in a spinal dog the reaction to a stimulus applied to the plantar surface of the hind foot differs in a way dependent on the nature of the stimulus; a firm gentle pressure causes extension, a sharp prick causes flexion. Sherrington apparently assumes the existence of one kind of nerve endings, nociceptors, which are excited by harmful stimuli, and which give rise to flexion, and another kind which respond to bland stimuli by extension. The analogy in the case of the dogfish is quite marked, except that the stimulus which is "bland" in one animal or in one state of the animal, is "nocuous" in another animal or in another state of the same animal.

There appears to me to be a yet closer analogy between these reactions and the reversibility of the heliotropic reactions of certain organisms; namely, those which are positive to weak and negative, to strong light. All the phenomena seem to me to indicate that the reversal of the stereotropic reactions of the dogfish is a central process. It has been objected to the idea that the heliotropic reversals are brought about in the nervous system that such reversals occur in unicellar organisms where no separate nervous system exists; but it has been pointed out by Loeb[4] that even in unicellular organisms

[3] Sherrington, Charles S., The integrative action of the nervous system, New Haven, 1906.

[4] Loeb J., Forced movements, tropisms, and animal conduct, Philadelphia and London, 1918.

structures could exist which would have the effect of synapses. It must be admitted, however, that notwithstanding the closeness of the analogy, the reversal of the heliotropic and of the stereotropic reactions may be due to totally different mechanisms.

SUMMARY.

1. The dogfish responds to certain contact stimuli by definite stereotropic reactions. These reactions can be positive or negative.

2. The sense of the stereotropic response depends on the strength of the stimulus; a "weak" stimulus, produces a positive and a "strong" stimulus, a negative response.

3. The strength of stimulus necessary to cause a reversal of the reaction depends in part on the physiological state of the animal.

4. The stereotropic reactions occur equally well in the absence of the forebrain.

CHEMICAL STIMULATION OF THE NERVE CORD OF LUMBRICUS TERRESTRIS.

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Nerve cells of different functions cannot be differentiated histologically by staining methods. In order to prove chemical differences it is necessary to affect the function of a given type of neuron by the action of a chemical agent. Thus Baglioni[1] has demonstrated a chemical difference between sensory and motor cells in the frog and the squid, by showing that phenol is an excitant for the motor ganglia alone while strychnine stimulates the sensory ganglia only of these forms. Maxwell[2] has shown that the nerve cells of the mammalian cortex are stimulated by one class of substances which includes creatine and strychnine, but that such substances do not act on medullated nerve fibers. Only certain salts, such as the calcium precipitants and barium compounds, act upon medullated fibers. On the other hand these salts do not stimulate the gray matter of the brain. Tetraethyl-ammonium chloride is an exception in that it acts upon structures of both types.[3]

It is possible that chemical differences exist between nervous systems of various forms.[4] For example, coelenterates give no spasm response to strychnine, but echinoderms do, provided the concentration of alkaloid is sufficiently high, while cephalopods are as sensitive to strychnine as vertebrates are. On these grounds Parker[5] has suggested that strychnine might be used as a test for the presence of synapses, since sensitivity to strychnine on the part of different

[1] Baglioni, S., *Z. allg. Physiol.*, 1905, v, 43.

[2] Maxwell, S. S., *J. Biol. Chem.*, 1906, ii, 183.

[3] Maxwell, S. S., *Am. J. Physiol.*, 1918–19, xlvii, 283. Loeb, J., and Ewald, W. F., *J. Biol. Chem.*, 1916, xxv, 377.

[4] Moore, A. R., *Proc. Nat. Acad. Sc.*, 1917, iii, 598.

[5] Parker, G. H., The elementary nervous system, Philadelphia, 1919, 208.

phyla develops hand in hand with increasing complexity of the nervous system.

By such a method it seems possible to discover similarities and relationships in the chemical constitution of neurons which would otherwise remain undetected. With this purpose in view experiments have been carried out on chemical stimulation of the nerve cord of the earthworm, *Lumbricus terrestris*.

In an experiment the animal was decapitated, pinned down by the anterior end and the anterior portion of the nerve cord laid bare for a distance of about 2 cm. A piece of cord about 1 cm. in length was then separated from the underlying tissue. The substance to be tested was applied to the loosened part. Stimulation was shown by spasmodic squirming of the posterior segments of the worm. Control experiments were made by applying the stimulating substance to a part of the body wall after removal of the nerve cord from that section of the worm. No reaction of the posterior segments resulted.

It is of course impossible to separate the nerve cells from their processes in this form. Therefore excitants of the first class,[2] *i.e.*, calcium precipitants and barium salts, were effective in causing stimulation. $BaCl_2$ and KCl in concentrations isosmotic with the worm's blood caused strong responses immediately. Responses due to the action of Na_2SO_4 and Na_3 citrate were weaker but unmistakable. Tetra-ethyl-ammonium chloride in M/64 concentration made up in Ringer solution acted as a powerful excitant.

Of the substances belonging to the second group, camphor in one-fifth saturated solution, strychnine in saturated solution, atropine sulfate in M/8 concentration and picrotoxin crystals all caused strong reactions. But creatine, caffeine, and nicotine phenol had no stimulating action. Creatine, caffeine, and phenol were applied to the nerve cord in the form of crystals and solutions; nicotine in concentrations of 0.004 per cent and 0.4 per cent was made up in isosmotic Ringer solution.

When chemical excitation did take place the response was almost immediate—within a minute of the time of application. This is a noteworthy fact since in chemical stimulation of the mammalian cortex the latent period is 10 or 15 minutes and the latent period for

the action of nicotine in squid is 6 or 7 minutes at 24°C., the concentration of nicotine being 1,500,000.

In conclusion the facts presented suggest, (*a*) that the nerve processes of *Lumbricus terrestris* are similar in chemical constitution to the axis cylinders of the medullated nerve fibers of mammals; (*b*) that the neurons of the earthworm are more limited in their possibilities of chemical stimulation; *i.e.*, they are simpler in chemical constitution, than the neurons of cephalopods and of mammals.

THE FORMATION OF THE ASTER IN ARTIFICIAL PARTHENOGENESIS.*

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In normally fertilized eggs the development of the aster is attributed to a substance carried into the egg by the spermatozoon. The aster first makes its appearance in the form of diminutive radiations surrounding the neck-piece of the spermatozoon within a few minutes after it has entered the egg. The writer[1] has shown that the formation of the radiations is accompanied by a jellying of the cytoplasm of the egg. The jellying process extends more and more as the aster increases in size and the entire egg becomes involved when the center of the aster comes to occupy the center of the egg.

The formation of the aster is accompanied by an increase in size of a hyaline area in its center. This is Wilson's hyaloplasm-sphere[2] also called centrosphere and astrosphere by other investigators. The microdissection method has demonstrated that this sphere area is liquid in contrast to the surrounding jellied cytoplasm. The pioneer observers of mitotic division, such as Auerbach, Hertwig, Bütschli and Fol, described the accumulation of a hyaline plasma at the astral centers and suggested that the astral radiations are a result of protoplasmic currents. Later investigators, such as Morgan, Wilson and Conklin, considered this view as the most probable one.

* The experiments, upon which this paper is based, were conducted in the Research Division of Eli Lilly and Company, at the Marine Biological Laboratory, Woods Hole. The experiments constitute a part of a joint research project in which Dr. G. H. A. Clowes and the writer are engaged.

[1] Chambers, R. Microdissection studies. II. The cell aster: A reversible gelation phenomenon, *J. Exp. Zool.*, 1917, xxiii, 483.

[2] Wilson, E. B., Experimental studies in cytology. I. A cytological study of artificial parthenogenesis in sea-urchin eggs, *Arch. Entwcklngsmechn.*, 1901, xii, 529.

The movement of the egg nucleus is possibly also a case in point. As long as the egg nucleus is beyond the confines of the aster it is stationary. As soon, however, as the extending aster reaches it, the nucleus begins travelling toward the sphere in which it finally lies close beside the sperm nucleus. The existence of a centripetal current may be inferred also from the following experiment. In an egg one may occasionally see one or more oil-like droplets 2 to 4 microns in diameter. If one of these droplets be pushed by the needle from the liquid cytoplasm into the periphery of the aster the droplet will move along the rays toward the center.

In view of the above observations it is highly probable that the liquid which accumulates in the center of the aster streams into it from all sides during the jellying of the cytoplasm. It is this streaming which probably occasions the innumerable radiations characteristic of the aster. After the aster has attained its full size the radiations begin to fade from view as the jelly state reverts to a more fluid one. The liquid of the central sphere does not mix with the fluid cytoplasm but separates into two areas, one at each pole of the mitotic figure of the dividing nucleus. Astral radiations now appear about the two areas as the egg cytoplasm jellies again with the formation of two jellied masses instead of one, as heretofore. These grow at the expense of the fluid cytoplasm until all of the cytoplasm of the egg is taken up into two bodies, the two blastomeres of the segmenting egg.

During the rapidly succeeding cleavages of the egg there is always a cap of liquid on the nucleus of each blastomere. With each mitosis this liquid flows around the nucleus to accumulate in two areas at the poles of the mitotic figure. These areas are periodically augmented during the formation of an aster and the ensuing jellying process.

There is every evidence[3] that the mechanism of cell division depends upon a readiness of the cytoplasm to pass from a liquid to a

[3] Heilbrunn, L. V., Studies in artificial parthenogenesis. II. Physical changes in the egg of *Arbacia*, *Biol. Bull.*, 1915, xxix, 149; An experimental study of cell division. I. The physical changes which determine the appearance of the spindle in sea-urchin eggs. *J. Exp. Zool.*, 1920, xxx, 211; Chambers, R., Changes in protoplasmic consistency and their relation to cell division, *J. Gen. Physiol.*, 1919, ii, 49.

jellied state and *vice versa*. The protoplasm must have its phase relations in a delicately balanced state in order that this may occur. In the egg we have seen that the reversal to a jellied state is probably accompanied by a separating out of a liquid. Something in this liquid may possibly control, in periodic rhythms, the physical state of the protoplasm surrounding it. We may assume that as long as there is a quantity of this substance localized in the egg it can induce aster formation. The idea suggests itself that one purpose of the spermatozoon is to accumulate this substance. In the mature unfertilized egg there is no localized area from which the jellying process may spread. The entrance of a sperm furnishes a focus as it were. Around this focus an aster develops with a steady accumulation of the liquid in its center. This liquid area surrounds the nucleus and puts the egg in a condition similar to that of a blastomere. The process of cleavage then becomes the same in both.

An interpretation disconsonant with previous ones concerning the mode of aster formation in artificially parthenogenetic eggs has been recently put forward by Herlant.[4] Wilson[2] in *Toxopneustes*, had long ago shown that eggs treated insufficiently with a parthenogenetic agent may form monasters which disappear and reappear in several successive rhythms. Hindle[5] found this to be true also for the sea-urchin egg, if treated with butyric acid alone. A sufficient treatment, however, of a parthenogenetic agent results in the disappearance of the monaster followed by the appearance of an amphiaster. This results in cleavage of the egg. In the sea-urchin egg, the butyric acid treatment has to be followed by a bath of hypertonic sea water in order that this may occur. The hypertonic treatment often results in the formation of several cytasters in the egg. The cytasters produced by the hypertonic treatment Herlant claimed to be due to dehydrative effects producing spots within the egg cytoplasm about which the asters appear. Herlant assumed that one of these cytas-

[4] Herlant, M., Comment agit la solution hypertonique dans la parthénogenése éxperimentale (méthod de Loeb). I. Origine et signification des asters accessoires. *Arch. Zool. exp. et gén.*, 1918, lvii, 511; II. Le mecanisme de la segmentation. *Arch. Zool. exp. et gén.*, 1919, lviii, 291.

[5] Hindle, E., A cytological study of artificial parthenogenesis in *Strongylocentrotus purpuratus*, *Arch. Entwcklngsmechn.*, 1910–11, xxxi, 145.

ters connects in some way with the monaster, thus forming the amphiaster which initiates segmentation. The weakness in this interpretation is the lack of conclusive evidence for the union of the originally independent asters. Neither Wilson nor Hindle ever observed such a phenomenon. All my observations also indicate that the amphiaster in parthenogenetic eggs arises from a previous single aster just as it does in normally fertilized eggs.

My studies were mainly confined to the egg of the sand-dollar. In its behavior to parthenogenetic agents[6] the egg is almost identical with that of the sea-urchin which Herlant studied. The absence of pigment and the highly translucent nature of its protoplasm makes the sand-dollar egg an ideal object for observational study.

The mature eggs, normally shed by the female, are placed in butyric acid (2 cc. 1/10 N in 50 cc. of sea water) for 35 seconds. During this treatment the eggs distinctly round up. They are then returned to sea water where, within a few minutes, the fertilization membrane lifts off. After 20 minutes the eggs are placed in hypertonic sea water (5 cc. 2.5 M NaCl in 50 cc. sea water). The eggs shrink slightly in this solution. After 20 minutes the eggs are transfered to a large quantity of normal sea water and the sea water is changed several times to free the eggs from any further action of the hypertonic solution.

Up to this time no change whatever is to be seen in the cytoplasm or in the nucleus. While in the hypertonic solution the cytoplasm appears more granular and opaque than that of an untreated mature egg. However, on the return of the treated eggs to sea water the cytoplasm reverts to its former appearance and to the eye the eggs differ in no respect whatever from unfertilized eggs except for the presence of a fertilization membrane.

It is not until the treated eggs have stood in sea water for several minutes that any cytoplasmic change is to be observed. The first sign of a change consists in the appearance of faintly defined vacuoles about the center of the egg. Within a few minutes they coalesce to form a central clear area of about one-tenth the diameter of the

[6] Just, E. E., The fertilization reaction in *Echinarachnius parma*. III. The nature of the activation of the egg by butyric acid. *Biol. Bull.*, 1919, xxxvi, 39.

egg. The egg nucleus lies close to or within this area. Gradually rays begin to appear in the jellying cytoplasm about the area. These rays become more numerous and more pronounced until the entire egg is occupied by a large monaster which corresponds exactly with the fully developed sperm aster of a normally inseminated egg. From now on the process is entirely analogous to that of a sperm fertilized egg. During the development of the aster the hyaline central area increases in size and the microdissection needle shows it to be a liquid area characteristic of that of the sperm aster. When the monaster disappears the liquid central area flows around the nucleus now undergoing mitosis and accumulates at the two poles of the nucleus into two polar areas. A jellying process now sets in with these two areas as centers and results in the amphiaster preparatory to the first cleavage of the egg.

In the mode of aster formation the only difference between the sperm fertilized and the parthenogenetic egg consists in the manner in which a liquid separates out of the jellying protoplasm in connection with the formation of the preliminary single aster. In the fertilized egg radiations appear immediately about the sperm-head and the accumulation of the liquid substance is from the beginning through the agency of the ray-like channels of the growing aster. In the parthenogenetic egg several vacuoles first appear in the cytoplasm. These vacuoles collect in the center of the egg after which an aster appears.

The frequent irregularities which obtain in parthenogenetic eggs are apparently due to an incomplete fusing of the vacuoles and to a lack of polarity in the preliminary stages of the aster formation. In undertreatment, or when butyric acid alone is used, a monaster developes as usual. Upon the disappearance of the monaster, the persisting liquid centrosphere, instead of flowing to the two polar regions of the nucleus, remains a single body. With the return of the jellying period a single aster again forms and more fluid accumulates in the centrosphere which increases in size. This process repeats itself several times and segmentation of the egg never occurs.

Eggs treated with butyric followed by a prolonged treatment of the hypertonic solution become abnormal. In cases of this kind the eggs, when returned to sea water from the hypertonic solution,

exhibit vacuoles which, instead of being collected in the center of the egg, are scattered throughout the cytoplasm. Radiations appear about these vacuoles with the result that the egg becomes filled with many small asters. The longer the eggs have been left in the hypertonic solution the more numerous will be the asters, and most if not all of these asters develop independently of one another. Irregularities may occur, even when the vacuoles collect in the center of the egg. In such cases an apparently normal single aster first results. Upon its disappearance, the central liquid area, instead of flowing away from the center into two polar bodies, produces three or four irregular lobes. About each of these lobes radiations appear in the egg cytoplasm producing a multipolar aster. In one instance one such lobe separated itself from the main body and a complete aster formed about it while a multipolar aster formed about the rest of the hyaline area. When the periphery of a multipolar aster reaches the surface of the egg cleavage furrows form between each lobe of the aster so that such eggs may segment simultaneously into three or four or more blastomeres. Asters which form independently of the central area never seem to be large enough to bring about segmentation of the egg into considerable masses. When such asters lie close to the periphery of the egg, furrows often grow in from the surface of the egg enclosing the asters. In this way a superficial type of segmentation results with the pinching off of small masses of the egg. The development of cytasters resulting in a spurious segmentation has already been described by Wilson.[2]

The first aster appears at about the same time after the acid treatment, irrespective of whether the eggs have been subsequently treated with the hypertonic solution or not. However, with subsequent hypertonic treatment, the reappearance of the radiations following the fading away of the first aster occurs about more than one center. This results in segmentation of the egg. The reaction, therefore, which is peculiar to hypertonic treatment shows up only *after* the disappearance of the first aster. At that time the persisting central liquid area of the aster, instead of remaining as a single centralized mass, separates into two or more bodies with the result that the following reappearance of rays in the cytoplasm occurs as radiations about these bodies. This produces multiple asters. If there be

only two focal points the liquid collects into two bodies, a typical amphiaster then develops, and the egg cleaves into two normal blastomeres.

Aster formation not only consists in a jellying process but also in the separating out of a liquid. The optically visible phenomenon peculiar to the parthenogenetic egg consists in the manner in which this liquid begins to separate out of the egg cytoplasm preparatory to the formation of the preliminary single aster. In the sperm fertilized egg both processes are rapid and occur together, radiations appear immediately about the sperm-head, and the accumulation of the liquid substance is from the very start through the agency of the ray-like channels of the growing aster. In the parthenogenetic egg the jellying process is apparently very slow, and the separating out of a liquid takes place before the cytoplasm is stiff enough to exhibit channels through which the liquid flows to the center. The liquid first collects into several vacuoles and an optimum treatment is necessary to cause these vacuoles to fuse into one body with the subsequent formation of a single aster. Overtreatment causes the appearance of many vacuoles scattered throughout the egg resulting in multiple asters. Undertreatment may result in the formation of a single aster which, however, periodically disappears and reappears as a single aster.

The parthenogenetic treatment, in order to be successful, must not only bring about the separating out of a liquid from the egg cytoplasm, but must also induce polarity within the resulting hyaline area in order to enable it to form two centers about which an amphiaster may develop.

STUDIES ON THE ORGANIZATION OF THE STARFISH EGG.*

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The following is a preliminary record of operative work on the starfish egg which throws some light on the nature of the fertilization membrane, the interaction between nucleus and cytoplasm, and the relation of the cortex to the interior of the egg.

By means of the microdissection needle it has been possible to show that a morphologically definite membrane closely invests the unfertilized egg, and that it is this membrane which lifts off upon fertilization as the so called fertilization membrane. The description of two methods will suffice to demonstrate this. By carefully pressing an unfertilized mature egg between the surface of a cover-slip and the side of a slender glass needle the egg may be cut in two without tearing the investing membrane. This membrane now becomes apparent, bridging the gap between the two egg fragments and holding them together. Upon the addition of sperm this membrane lifts off as the fertilization membrane, in such a way that the two egg fragments come to lie within a single cavity.

The unfertilized egg can also be slipped entirely out of its investing membrane. Such an egg will undergo normal fertilization and cleave into blastomeres having no investing membrane whatever.

These two experiments definitely show that the normal unfertilized starfish egg is already surrounded by a membrane which, upon fertilization, becomes the fertilization membrane.

The difference in behavior towards sperm of an egg, which has been denuded not only of its jelly but also of its membrane, and one which has not is very striking. In an egg enclosed in its membrane

*The experiments reported in this paper constitute a part of the joint investigation of the mechanism of fertilization in which Dr. G. H. A. Clowes and the writer are engaged.

the spermatozoa quickly crowd about the egg as they are trapped in the jelly surrounding the membrane. In a membraneless egg no crowding of spermatozoa is noticeable and heavy insemination is necessary to bring about fertilization. With such eggs, when a cloud of sperm has been blown upon them, one may frequently observe a spermatozoon swim toward an egg, wander over its surface and then swim away. On the other hand the empty membrane with its investing jelly immediately becomes covered with a halo of active spermatozoa.

The nucleus of the egg cell is a liquid drop surrounded by a morphologically definite membrane. The nucleus may be moved about within the egg with the needle, and can be considerably deformed by pressure. On removal of the needle the nucleus quickly resumes its spherical shape. Tearing the nucleus slightly causes the nucleus to shrink and the nucleolus to disappear; this is followed by a remarkable spread of a disintegrative process which involves the cytoplasm surrounding the nuclear area. In the immature egg, where the nucleus is large, the disintegrative process may extend throughout the entire egg. In the mature egg with a relatively small nucleus the destruction is restricted to a limited area.

The disappearance of the nucleus or germinal vesicle during maturation has been described by several investigators. The nuclear membrane breaks down spontaneously and the nuclear sap spreads slowly throughout the cytoplasm. So long as the nuclear area, aside from the definitive egg nucleus, has not yet mixed with the cytoplasm, I find that a puncture of the area starts up the disintegrative process. When the nuclear sap has entirely mixed with the cytoplasm, any part of the egg, with the exception of the minute egg nucleus, may be torn with impunity. The mere presence of the glass needle in the nuclear sap is not sufficient to start up the disintegrative process. This process occurs only when the nuclear sap is agitated by the needle while the sap is in direct contact with the cytoplasm.

Wilson[1] found in the Nemertine egg that any non-nucleated fragment, prior to the dissolution of the germinal vesicle, is non-fertilizable whereas, any fragment from a mature egg is capable of being fertilized and undergoing cleavage. This I have found to be true also for the

[1] Wilson, E. B., Experiments on cleavage and localization in the Nemertine egg, *Arch. Entwcklingsmechn.*, 1903, xvi, 411.

starfish egg. It is also of interest to note that the fertilizability of the egg fragments is directly connected with the extent of the mixing of the nuclear sap with the cytoplasm in the maturing egg. A non-nucleated fragment, taken from an egg in the early stages of the dissolution of the germinal vesicle, will admit sperm which will undergo several nuclear divisions with, at most, an abortive attempt on the part of the fragment to cleave. When the sap of the germinal vesicle has completely mixed with the cytoplasm, any fragment larger than a certain size limit is capable of being fertilized and undergoing cleavage.

It is well known that immature eggs can be kept in sea water at room temperature for 24 hours or more without disintegrating and that unfertilized mature eggs go to pieces under the same conditions within a much shorter time.[2] The writer has found that nucleated fragments of the two kinds of eggs behave similarly, while non-nucleated fragments act quite differently indicating that the substance which prevents the disintegration is distributed differently in the two eggs. Non-nucleated fragments of immature eggs last for about 4 hours only. Similar fragments of mature eggs last from 8 to 10 hours, or about as long as the mature, nucleated fragments. The substance which prevents the destruction of the egg is apparently in the nuclear sap which, in the immature egg, is confined within the large nucleus or germinal vesicle, while in the mature egg this sap has escaped from the nucleus and spread throughout the entire egg.

The following experiments indicate that the part of the starfish egg which is capable of development is chiefly confined to the cortex of the egg. It was long ago shown by Driesch,[3] Loeb[4] and others that starfish and sea-urchin eggs are highly fluid in that fragments quickly round up into spheres. That the cortex of the mature unfertilized eggs is firmer in consistency than their interior has been

[2] Loeb, J., and Lewis, W. H., On the prolongation of the life of the unfertilized eggs of the sea-urchins by potassium cyanide, *Am. J. Physiol.*, 1902, vi, 305. Loeb, J., Maturation, natural death and the prolongation of the life of the unfertilized starfish eggs (Asterias forbesii) and their significance for the theory of fertilization, *Biol. Bull.*, 1902, iii, 295.

[3] Driesch, H., Entwicklungsmechanische Studien. Der Werth der beiden ersten Furchungszellen der Echinodermentwicklung, *Z. wiss. Zool.*, 1891, liii, 60.

[4] Loeb, J., Ueber die Grenzen der Theilbarkeit der Eisubstanz, *Arch. Physiol.*, 1895, lix, 379.

described by the writer.[5] If the surface of the mature starfish egg be torn with a needle, and the egg then caught at the opposite side and pulled to the edge of the hanging drop, the compression on the egg produced by the shallow water at the edge of the drop will cause the fluid interior to ooze out through the tear, forming a perfect sphere. One may so manipulate the process as to cause the egg nucleus either to remain behind in the cortex (the cortical remnant) or to pass into the extruded sphere.

The cortical remnant is relatively solid and remains more or less enclosed within the egg membrane and its jelly. If left long enough it will eventually round up so as to present the appearance of a diminutive egg surrounded by a collapsed and wrinkled egg membrane.

The material which has escaped from the egg into the sea water is fluid and tends immediately to round up. On tearing with a needle its surface behaves like that of a highly viscous oil drop. These spheres adhere tenaciously to glass and, in the effort to remove them by blowing a current of water against them, they sometimes leave a torn off piece behind. The cortical remnant is readily fertilizable and undergoes normal segmentation. On the other hand, the material which has escaped from the interior of the egg whether nucleated or not, is non-fertilizable. It remains inert until it finally undergoes disintegration. As long as it possesses an intact surface it appears exactly like an egg fragment and will undergo disintegrative changes similar to those of entire eggs, on being torn with the needle. If even a small part of the original cortex is allowed to remain continuous with the sphere it is fertilizable and the more cortical material present the more will the sphere approach normal cleavage.

It is significant that the fluid spheres which escape from the interior of the mature unfertilized egg, whether nucleated or not, withstand disintegration for a much longer period than do fragments, containing cortical material, which have been produced simply by cutting an egg into two or more pieces.

It follows from these facts that the part of the starfish egg chiefly concerned in development lies in its periphery. The interior when separated from the cortex is incapable of developing. On the other hand, an egg containing cortical material alone is able to carry on its usual life activities.

[5] Chambers, R., Microdissection studies. I. The visible structure of cell protoplasm and death changes, *Am. J. Physiol.*, 1917, xliii, 1.

THE SELECTIVE ABSORPTION OF POTASSIUM BY ANIMAL CELLS.

I. Conditions Controlling Absorption and Retention of Potassium.

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The accumulation of potassium with little or no sodium in the cell living in a medium richer in sodium than in potassium is so widespread a phenomenon as to be considered a general attribute of living cells. In the few exceptions on record the distribution of sodium between cells and tissue fluids has not been satisfactorily investigated. The mechanism governing potassium selection has not been explained. Loeb (1) as early as 1906, stated, "we may take it for granted that, at least, potassium forms a non-dissociable constituent of the protoplasm of a number of tissues of animals and plants." The view that much of the potassium of the cell is not dissociated seems generally accepted. Robertson (2), for example, uses the selective action of cells for potassium as one of the proofs of the dissociation of protein salts so as to hold inorganic constituents as a part of protein ions without the formation of free inorganic ions. Such a theory explains very well the retention of accumulations of potassium in the cells but it gives no clew as to why potassium is "selected" instead of sodium nor, in the face of the apparent impermeability of normal cells to electrolytes, does it explain how potassium makes its way into growing cells. Our investigations bear on such aspects of the problem.

It seemed of first importance to find some of the limiting conditions for the retention of potassium. The work of Loeb (3), showing the so called salt effect as necessary for both the inward and outward diffusion of potassium from the fertilized eggs of *Fundulus*, does not apply to such a structure as a muscle cell. It does show that the presence of a certain amount of salt or of acidity or both is necessary

for the passage of potassium through the egg membrane and that sufficiently prolonged action of pure water makes this membrane very impermeable to potassium. But, as Loeb points out, the behavior of the hatched embryo is very different from that of the egg. In the egg we are dealing merely with the passage of potassium through a comparatively distinct membrane but in the case of most cells we must consider the taking up of potassium, whatever that means physicochemically, by the cell acting as a whole. Salts are always present and acidity absent in the medium bathing the great majority of living cells. Hamburger (4) has shown that blood corpuscles, washed with glucose solution, yield potassium to a potassium-free Ringer solution, and Jannink (5) has reported that potassium is given off by heart muscle during perfusion with a potassium-free solution. Howell and Duke (6) recorded a liberation of potassium from the heart perfused with Ringer solution during prolonged stimulation of the vagus. These and other experiments, though they point out interesting possibilities, do not reach the explanation of the conditions limiting the entrance or exit of potassium, considering cells in general.

Muscle seemed a favorable material to use because it gives an easily controlled range of physiological activity. The work of Lillie and of McClendon indicates that, under some circumstances at least, contracted muscles are more permeable than resting ones. Is this true for potassium? We sought the answer to the question by making determinations of the amount of potassium in frog muscles after perfusion with various solutions either with or without simultaneous excitation. The results have shown the great tenacity with which the cells hold potassium while bathed in a potassium-free Ringer solution irrespective of whether or not the muscles are made to contract. As soon, however, as the muscles are fatigued beyond physiological limits potassium diffuses out of the cells and as much as half of their store may be lost in about 5 hours. Since potassium is known to diffuse slowly from dead muscle and since extreme fatigue in excised muscle is an irreversible process this was to be expected. A study of the intermediate stage between mild activity and exhaustion, showed that a part, from 8 to 15 per cent, of the potassium may be removed by perfusion, either with or without stimulation, without

marked loss of irritability in the muscle substance. Our experiments though not including any quantitative measurements of irritability have shown to rough observation a progressive loss of excitability accompanying potassium depletion. This is to be expected from the well known contrast between the physiological effects of a potassium-free physiological saline and those of Ringer solution.

We have further undertaken to test the possibility that muscular contraction is favorable to the process by which potassium is absorbed. We have found that only a contracting muscle, in sharp contrast to a resting one, can take up rubidium and cesium, substances whose chemical properties are more like those of potassium than sodium, so as to retain them in the same sense that potassium is retained.

EXPERIMENTAL.

Large bull frogs weighing from 125 to 400 gm. were used. Some of them were collected in Louisiana in February, shipped in March and used for the experiments in April, May and June. Others were taken in Rhode Island or Massachusetts in summer and used shortly after collection. Perfusions were made through a glass canula in the dorsal aorta of the pithed animals. The muscles used for analysis were lightly drained on paper after removal from the animal and immediately weighed. After digesting the muscle in a mixture of nitric and sulphuric acids potassium determination was made by the method described by Clausen (7) for blood analysis. Muscles removed from frogs without experimentation, merely killing and bleeding the animal, gave results as shown in Table I.

The averages of these results, whether computed for the same muscles of different frogs or for the various muscles of the same frog, are fairly uniform and are in agreement with averages obtained by Fahr (8) and others, placing the normal content of potassium in frog muscle at 0.34 per cent. The considerable variation in the results is partly due to errors in sampling. The sartorius is too small in many frogs for a satisfactory analysis. The vastus cannot be removed intact in its sheath so as to yield clean cut prisms of muscle but is apt to suffer loss of some of its fluids during sampling. For this reason most of the experiments reported below were done with the gastrocnemius muscles which can be more satisfactorily sampled. Another

and more important cause of variation is due to fluctuations in the water content of the muscles. In a series of nineteen determinations of the water in fresh (not perfused) frog muscles the minimum was 79.10 per cent and the maximum 82.22 per cent. Comparison of these results with the previous history of the animals, season when collected, duration of captivity, etc., showed no consistent correlation. The variation is sufficient, however, to make it advisable, when comparing the potassium content of different muscles, to determine potassium as per cent of the dried weight of the muscle. The average for the series was 80.78 per cent which is within the range of the averages commonly given for water content of muscles of cold-blooded animals. A third, and chief reason, is the actual variation in the

TABLE I.

Potassium Content of Fresh Normal Frog Muscle.
Results Are in Per Cent of Potassium in Moist Muscle as Weighed.

Muscle.	First frog.		Second frog.		Third frog.		Average.
	Left leg.	Right leg.	Left leg.	Right leg.	Left leg.	Right leg.	
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Gastrocnemius	0.330	0.322	0.355	0.362	0.331		0.340
Sartorius	0.370	0.342	0.337	0.378	0.328	0.305	0.343
Vastus	0.328		0.300	0.327	0.348	0.349	0.330
Average	0.338		0.343		0.332		0.338

potassium content of the muscles of different frogs. For example the average of twelve analyses on eight frogs taken from localities in Rhode Island and Massachusetts during the summer and analyzed soon after collection was 1.807 per cent of potassium in the dried muscle while the average of seven analyses on muscles of six frogs brought from Louisiana in winter and used some months later was 1.591 per cent of potassium in dried muscle. The relation of the potassium content of frog muscle to reproductive activities, to sex, to the food supply, and to species would be interesting. Such studies have not been attempted in this work. The results indicate that a prolonged stay in an aquarium without food causes a diminution of potassium. The individual variations are greater than the analyti-

cal error and are due to something more than a mere seasonal variation since in a group of twelve analyses on eight summer frogs the maximum variation was 17 per cent of the average potassium content of the dried muscle while the analytical error as indicated by duplicate analyses of the same muscle or by analyses of the two fresh gastrocnemii, of the same frog did not exceed a maximum of 2.3 per cent and averaged 1.37 per cent.

The effect of perfusion with a potassium-free Ringer solution on the potassium content of muscle was contrasted with that of similar perfusion with Ringer solution. In each case both legs were perfused during 15 minutes with about 300 cc. of solution until the venous outflow appeared bloodless; the artery of one leg was then tied

TABLE II.

The Potassium Content of Perfused Muscles.
Results Are in Per Cent of Potassium in the Moist Muscle, as Weighed.

Muscle.	Perfused with potassium free solution.		Perfused with Ringer solution.	
	Slightly perfused. 300 cc.	Much perfused. 1800 cc.	Slightly perfused. 300 cc.	Much perfused. 1800 cc.
	per cent	*per cent*	*per cent*	*per cent*
Sartorius	0.263	0.240		
Vastus	0.275	0.222	0.292	0.248

off and 1500 cc. of solution perfused in $1\frac{3}{4}$ hours through the other leg. Results are given in Table II.

The potassium content of the moist muscle was decreased in both experiments. Fahr (8) has shown that frog muscle immersed in Ringer solution during 20 hours contained a smaller percentage of potassium than fresh muscle, though after a similar immersion in isotonic sugar solution a greater potassium loss was shown. The lower per cent of potassium obtained after perfusion seemed attributable in part, at least, to the higher water content. This possibility was indicated by the obvious distention and edematous appearance of the much perfused leg. Edema as a result of perfusion with a number of physiological salt solutions has been observed by Guntzberg (9), Abel (10), and others. Another experiment was therefore

tried with determinations of water as well as potassium in the muscles. After perfusing 200 cc. of a potassium-free Ringer solution through both legs, one leg, was tied off and 800 cc. were then perfused in the course of 8 hours through the other leg. The gastrocnemius of the slightly perfused leg yielded 0.304 per cent of potassium and the same muscle of the other leg 0.267 per cent; but the first of these muscles contained 18.85 per cent of solids while the second had only 16.7 per cent, so that the slightly perfused muscle contained 1.61 per cent of potassium in its solids and the more extensively perfused one had 1.60 per cent of the solid matter. The average content of potassium in the solids of the fresh muscle is 1.75 per cent, so that muscles of both legs had doubtless lost some potassium. These experiments were done before the extent of variability in water and potassium content of muscles of different frogs was fully appreciated. In a further series samples of the gastrocnemius of one leg were taken after a brief perfusion, lasting less than 10 minutes, sufficient to remove all visible blood. The muscles of the other leg were then perfused during a suitable period and samples of both gastrocnemii, after vacuum desiccation to constant weight, were used entire for wet ashing and potassium titration. The effect on the perfused muscle was then computed as the percentage loss of the original potassium content as shown by the muscle that was not perfused. Such experiments gave consistent results and showed, as set forth in Table III, a loss of potassium during early stages of perfusion with the limit of potassium loss approached after 5 hours. Subsequently little or no loss of potassium occurs. The time of perfusion showed more consistent relation to potassium loss than did the amount of perfusion solution used. This indicates diffusion of potassium from the muscle cells into the fluid of the lymph spaces irrespective of considerable change in the rate at which the latter is replaced. Such a result may be explained by the fact that in any case the perfusion solution, present in such quantities as to greatly distend the lymph spaces, would be notably lower in potassium content than the intracellular fluid.

The relation of muscular activity to loss of potassium from the muscle cells was sought in the following experiment. Potassium-free Ringer solution was perfused through both legs during 2 hours.

500 cc. of solution were used. During the last 1½ hours the muscles of one leg were stimulated intermittently with strong induction shocks applied to the distal end of the severed lumbar plexus. The average potassium content of the muscles of the rested leg was then found to be 0.350 and of the stimulated muscle 0.306 per cent. In another experiment 2½ liters of solution were perfused and stimulation with intermittent rest periods was applied during 2½ hours to one leg. The average potassium content of the muscles of the rested leg

TABLE III.

Loss of Potassium from Muscles Perfused with Potassium-free Ringer Solution.

Total time of perfusion.	Total amount of solution used.	Average rate of perfusion.	Solids in control muscle.	Potassium in moist control muscle.	Potassium in solids of control muscle.	Solids in perfused muscle.	Potassium in moist perfused muscle.	Potassium in solids of perfused muscle.	Apparent loss of potassium.*
hours	*cc.*	*cc. per minute*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
7	800	1.9	18.69	0.376	2.016	15.65	0.276	1.765	12.5
8	900	1.9	19.51	0.336	1.879	14.73	0.243	1.648	12.3
12	1800	2.5	18.87	0.335	1.775	16.20	0.245	1.511	14.8
13¾	1500	1.8	18.96	0.335	1.765	14.25	0.214	1.499	15.1
1¼	100	1.3	19.20	0.281	1.465	16.97	0.237	1.399	4.5
5⅓	800	2.4	20.60	0.292	1.417	17.97	0.233	1.298	8.3
14¼	1000	1.2	18.75	0.313	1.670	14.64	0.225	1.537	8.5
18¾	1600	1.4	18.56	0.309	1.667	15.48	0.237	1.532	8.1

*Figures in this column are obtained, as explained in the text, by computing the difference between potassium in solids of control muscle and in those of perfused muscle as per cent of the potassium in the solids of the control muscle.

The frogs used in the first four experiments reported in this table were collected in summer and used shortly after they were brought to the laboratory. Those used in the last four experiments were collected in winter in Louisiana and used some months later.

was 0.299 per cent and of the stimulated leg 0.237 per cent. The stimulated muscles show lower potassium content than the resting ones. Siebeck's (11) observation, confirmed by Meigs and Atwood (12), that a muscle in isotonic potassium chloride solution takes on more weight if active than if at rest should be recalled in this connection. The apparent losses of potassium in the stimulated as contrasted with the rested muscles are not, indeed, greater than could be accounted for as percentage changes due to absorption of

water. We hoped to avoid this effect and produce more nearly physiological conditions by the use of gum arabic in the perfusion solution, employing, in short, a modified Bayliss solution. Gum arabic, purified by six alcohol precipitations from dilute hydrochloric acid solution, and showing by analysis an amount of potassium less than 0.01 per cent was used. The solution contained the following:

NaCl	6.5 gm.
$CaCl_2$	0.25 "
$NaHCO_3$	0.20 "
Gum arabic	45 "
Phenol-sulphonephthalein	a few drops.
Water	1 liter.

It had a pH of approximately 7.3; 700 cc. of the solution were perfused in $6\frac{1}{2}$ hours through both legs. The muscles of the left leg were stimulated through the lumbar plexus with maximal tetanizing induction shocks lasting 1 second each, given by an automatic stimulator at 30 second intervals during the first half of each hour with half hour rest periods intervening. These muscles, then, made 360 contractions lasting 1 second each. The muscles of both legs showed, at the end of the experiment, good though not equal irritability. The average potassium content of the muscles of the stimulated leg was 0.229 per cent, of the muscles of the other leg, 0.228 per cent. Both of these figures are lower than the percentages of potassium found in normal frog muscle. Both legs showed, however, some swelling in spite of the gum arabic. To determine the effect of contraction on the potassium content it seemed necessary to compute it in relation to the solids of the muscle. A further experiment for this purpose was undertaken. 900 cc. of potassium-free Ringer solution were perfused through both legs in $8\frac{1}{2}$ hours. Gum arabic because of the labor involved in making it potassium-free was not used. The muscles of one leg, stimulated by the method described for the preceding experiment, gave 480 contractions, of 1 second each. The muscles of both legs showed good irritability at the end of the experiment. The average potassium content of the muscles of the stimulated leg was 0.203 per cent, of the muscles of the other leg, 0.230 per cent, but computed as percentages of the solids: 1.86 per cent for the former and 1.85 per cent for the latter. No especial loss of potassium due to stimulation was shown.

In addition, the experiment reported on the second line in Table III included stimulation by the same method so that the muscles in the course of 8 hours of perfusion gave 330 contractions of 1 second each. The results of this experiment do not fall out of line with those including no stimulation. Results therefore give no indication of loss of potassium while the muscle is contracting, other than the loss attributable to the presence of a potassium-free medium.

Such results are in marked contrast to those obtained when muscle is stimulated enough to cause loss of irritability. The considerable difference between the potassium content of the rested and the exhausted muscle as found in the following experiment seemed too great to be explained on the basis of change in muscle weight. Both legs were perfused with potassium-free Ringer solution during 2 hours. The muscles of one leg were given prolonged tetanizing stimuli at first through the nerves until they failed to respond and then by direct stimulation until exhausted. The average potassium content of the muscles of this leg was 0.141 per cent and that of the rested muscles, 0.266 per cent. A similar experiment to determine the potassium content of the muscle *solids* after exhaustive stimulation was made. 1600 cc. of potassium-free Ringer solution were perfused through both legs in $6\frac{1}{4}$ hours. The muscles of one leg were given direct tetanizing stimuli for varying periods with intervening rest. The total time of stimulation was 98 minutes. The average potassium content of the muscles of this leg was 0.102 per cent; of the others, 0.221 per cent. Computed as percentages of the solids there was 0.89 per cent in the fatigued and 1.70 per cent in the rested muscles. The stimulated muscles lost about half of their potassium.

The development of acidity in a fatigued muscle suggests that hydrogen ion concentration is a factor in the control of the processes by which potassium is retained or set free in the cells. Experiments to test this possibility will be the subject of a later communication. For the experiments reported in this paper perfusion solutions were in general adjusted with sodium bicarbonate, using Clark and Lubs (13) indicators, to a pH of approximately 7.3.

That muscular contraction does enable the cells to *take in* potassium is indicated by experiments on the absorption by muscles of rubidium and cesium, substances very similar to potassium in chemical and

physiological behavior. This work is reported in another paper (14), but may be briefly summarized in so far as it applies to the subject under discussion. It included experiments in which frog's legs were perfused with a Ringer solution whose potassium chloride was replaced by equivalent amounts of rubidium or cesium chloride. The muscles of one leg were meanwhile stimulated to contract without complete fatigue. Subsequently both legs were perfused with a potassium-free Ringer solution until the outgoing fluid gave no spectroscopic test for rubidium or cesium. The muscles of both legs were then analyzed for rubidium or cesium. Only those of the leg which had been stimulated during the first perfusion showed the presence of these metals. To take them, and therefore their close homologue, potassium, into the muscle in such manner as to be retained, in a non-diffusible form would seem to require contractile activity of the muscle.

DISCUSSION.

These experiments indicate that, as Loeb (1) has suggested, the potassium of muscle cells can be considered as existing in two different states. The portion constituting one of them is comparatively mobile, consists of about 15 per cent of the muscle potassium in summer frogs and a smaller proportion in winter frogs, and can be removed from frog muscle, under the conditions described, by mere perfusion with a potassium-free Ringer solution. The rate of its loss seems to be unaffected by contractile activity of the muscle. The remainder of the potassium, amounting to approximately 15 per cent of the dry solids of a perfused muscle, markedly resists outward diffusion but is liberated and rapidly lost when the muscle is stimulated to an extreme stage of fatigue. That some deep-seated change in the chemical composition of a muscle occurs during unusually prolonged exertion is evident from the long duration of the after effects. That the change causes an alteration of the conditions governing the partition of inorganic constituents between dissociable and non-dissociable forms is suggested by these observations. The greater osmotic pressure of fatigued muscle as compared with non-fatigued was shown by Moore (15) and taken to indicate an increased concentration of electrolytes in the cell during contraction unless they are removed by the circulation. The work of Loeb (16) showing the absolute

dependence of the formation of metallic salts of proteins on hydrogen ion concentration is helpful in forming a conception of the behavior of potassium. If one believes with Robertson (2) that salts of the proteins may be so built that the inorganic constituent is, partly at least, in non-dissociable form then one can readily conceive of how an intracellular change of hydrogen ion concentration such as occurs in a fatigued muscle would markedly alter the distribution of potassium, and doubtless other inorganic constituents, between the dissociable and the non-dissociable forms.

As to the absorption of potassium a conception of how contraction might be essential to the mechanism involved is difficult. One notes the generally accepted idea that ion movements having definite relation to membranes are concerned in excitation. This idea is, of course, the basis of the Nernst theory of stimulation and is assumed in the conception of the mode of propagation of excitation as developed by Lillie (17). Because ions move during excitation and perhaps penetrate membranes, are we to assume that the potassium ion can penetrate the cell only at that time? In the light of our present knowledge it seems necessary to believe that cell permeability is not merely a matter of passage through an external membrane but that the physiological behavior of the protoplasm as a whole must be considered. The work of Wishart (18) on the distribution of glucose between plasma and corpuscles may be cited as one example of such observations. If contraction, involving migration of ions, causes a sort of fixation of rubidium or cesium within the cell it is fair to suppose that potassium would be similarly affected. That potassium favors excitability by its presence at cell surfaces has been amply demonstrated for a variety of tissues, so that some significant movement of potassium ions is involved, among other things, in a response such as muscular contraction. Our results suggest that movement at the cell surface is not the only factor concerned but that some transformation of potassium into a non-dissociable form occurs, presumably within the protoplasm. In this connection the work of Crozier (19) on sensory activation of cells by acids is of interest since it led him to the view that stimulation does not merely involve an increase in permeability but that the change of condition of materials at the surface of cells is instrumental in determining diffusion of ions within the cell.

SUMMARY.

1. Individual variations in the potassium content of the fresh muscles of frogs are notable even when computed as percentages of the dry solids. The potassium content averaged higher in freshly collected summer frogs than in winter frogs after a period of captivity.

2. Muscles show a loss of from 8 to 15 per cent of their potassium during perfusion with potassium-free Ringer solution but tenaciously hold the remainder.

3. Muscles, stimulated to contract under conditions that do not produce irreversible stages of fatigue, show losses of potassium no greater than those attributable to the presence of a potassium-free medium.

4. A condition favorable to the taking up of potassium probably occurs in a contracting muscle because rubidium and cesium, substances very similar to potassium in chemical and physiological behavior, are absorbed in retainable form by a contracting muscle but not by a resting one.

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COMPARATIVE HYDROLYSIS OF GELATIN BY PEPSIN, TRYPSIN, ACID, AND ALKALI.

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Gelatin and proteins in general may be hydrolyzed by either acids (hydrogen ions), alkali (hydroxyl ions) pepsin, or trypsin. In all cases the reaction consists in the rupturing of one or more of the peptide linkings with the addition of 1 molecule of water. The ease with which the various linkages are split is very different, as was shown by Fischer,[1] in his study of the hydrolysis of the polypeptids. The same phenomenon is shown by the fact that the products of partial hydrolysis of the proteins form a series of increasing complexity—a result of the fact that some of the linkages are much more easily split than others. The same fact is brought out by following the course of the reaction in strong acid for example. If all the linkages were split at the same rate, the reaction would follow the course of a monomolecular one. This is not the case. The velocity decreases steadily as the reaction proceeds, showing that some of the linkages are split more easily than others.

The question arises then as to whether those linkages which are most easily split by acid, for instance, are also the most easily split by pepsin and trypsin. There is evidence already that this is not the case. Henriques and Gjaldbäk[2] have shown by an ingenious modification of the formol titration that protein solutions having the same total content of titratable amino or carboxyl groups but which have been brought to this stage by different hydrolytic agents, may be distinguished from each other by their behavior when titrated with alkali to different end points, showing that, although the total

[1] Fischer, E., Untersuchungen über Aminosäuren Polypeptide und Proteine. Berlin, 1906.

[2] Henriques, V., and Gjaldbäk, J. K., *Z. physiol. Chem.*, 1911, lxxv, 363; 1913, lxxxiii, 83.

number of linkages split was the same in all the solutions, different ones had been split in each case. They found that the course of the reaction was similar with pepsin and acid, and with trypsin and alkali. The study of the hydrolytic products of trypsin and pepsin leads to the same conclusion.[3] It is usually stated that trypsin hydrolysis leads to more amino-acid formation.

More quantitative evidence may be obtained, however, by a slightly different mode of procedure; namely, by adding the enzyme in question to a solution of the protein which has already been partially hydrolyzed by some other means and noting the subsequent increase in hydrolysis. If the two reactions follow the same course, *i.e.*, if the same linkages are split in both cases, the final amount of hydrolysis will be the same irrespective of the stage at which the enzyme is added. If, however, the preceding hydrolysis has split the protein at a linkage which is not attacked by the enzyme, then the total amount of hydrolysis will be the greater the farther the hydrolysis had proceeded before the enzyme was added. That is, the total hydrolysis will be equal to the sum of the enzyme hydrolysis (on the unhydrolyzed protein) plus that which had taken place due to the other hydrolytic agent. Since it is known that acids and alkali are capable of hydrolyzing the proteins to their constituent amino-acids, it is obvious that they must be able to split all the linkages so that a stage must eventually be reached in acid or alkali hydrolysis at which the addition of the enzymes will cause no further hydrolysis. The stage in the hydrolysis at which this occurs will evidently be a measure of the difference in the course of the two reactions. If the linkages which are most easily split by pepsin, for instance, are the ones which are most slowly attacked by acid, it will evidently be necessary to almost completely hydrolyze with acid the protein before reaching a stage at which the addition of pepsin will cause no further increase. As will be seen below, this is really the case.

The results are complicated by the fact that the ease of hydrolysis depends on other factors as well as on the particular linkage which is hydrolyzed. This was shown by Fischer[1] who found that tetraglycyl glycine may be hydrolyzed by trypsin whereas triglycyl glycine is not

[3] *Cf.* Fischer.[1]

hydrolyzed. It is, therefore, not possible to conclude that the enzyme ceases to act, when a certain stage of the hydrolysis has been reached, because all the linkages which it is capable of hydrolyzing have already been split. The molecule may have been modified in some other way and so become resistant to the action of the enzyme although still containing a linkage which under certain conditions may be attacked. The same influence is undoubtedly also shown on the rate of hydrolysis by acids or alkali. There does not seem to be any evidence to distinguish qualitatively between the specificity of an enzyme and of hydrogen ions.

The procedure outlined above was followed in the experiments described in this paper. The gelatin was hydrolyzed to various stages by one of the hydrolyzing agents mentioned, pepsin or trypsin then added, and the increase in hydrolysis noted. The reaction was followed by means of a slight modification of the formol titration.

EXPERIMENTAL.

Gelatin.—Powdered gelatin, purified by washing at the isoelectric point,[4] was used in all the experiments. The solutions were made up to contain 5 gm. of gelatin per 100 cc.

Pepsin.—The pepsin used was an active preparation of Fairchild Bros. (U. S. P. 1/19,000). It was prepared for use by dissolving 5 gm. in 100 cc. of water, adjusting to pH 2.5 with HCl and dialyzing under pressure against 0.01 N HCl for several days. The solution thus obtained had a very low titration which remained constant. In the concentration used the correction for the pepsin solution was negligible. The solution is referred to as 5 per cent pepsin.

Trypsin.—Fairchild's trypsin was used. It was prepared for use by dissolving 10 gm. in 100 cc. of water and dialyzing under pressure against running tap water at 6°C. for about 18 hours. The solution is very unstable and loses its activity in 3 or 4 days even at 2°C. The formol titration of this solution was negligible in the concentrations used and remained constant. This solution is referred to as 10 per cent dialyzed trypsin.

Hydrogen ion determinations.—The determinations were made by the E. M. F. method.

Formol titration.—The titration was carried out as described in a previous paper.[5] The method consists essentially in freeing the solutions from CO_2 by adjusting the pH with acid, and boiling for a few seconds. The solution is then

[4] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 237.

[5] Northrop, J. H., *J. Gen. Physiol.*, 1920–21, iii, 715.

accurately adjusted to pH 7.0, using neutral red as an indicator. The formaldehyde is then added and the titration carried out as usual with 0.1 N NaOH, using thymol blue as indicator and an end-point of about 8.2. The titration was made on 10 cc. until the titration figure became more than 5 cc., after which 5 cc. were analyzed. The results have been calculated on the basis of 10 cc. of 5 per cent gelatin. The titration value for this concentration of gelatin was found to be 2.0 cc. The complete hydrolysis of gelatin increases the amino nitrogen (or formol titration) about 20 times.[5, 6] The percentage total hydrolysis may therefore be found from the figures by dividing by 40.0.

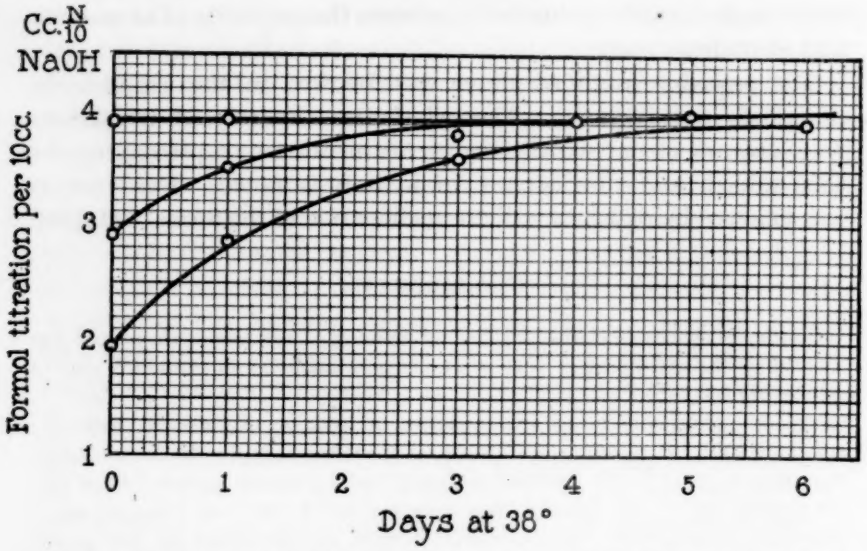

FIG. 1. Hydrolysis of gelatin by pepsin. 500 cc. of solution containing 25 gm. of gelatin, 20 cc. of 5 per cent dialyzed pepsin, and 40 cc. of 1.0 N HCl placed at 38°C. (pH = 2.2). 50 cc. samples pipetted out after 0, 1, and 4 days, and kept at 3° until all had been taken. 5 cc. of 5 per cent pepsin added to each sample and the samples placed at 38°C. Formol titration was run on 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

Hydrolysis by Pepsin Alone.

The results of this experiment are given in Fig. 1. The figure shows that under the conditions of this experiment, the pepsin is able to double the formol titration of the gelatin (an increase of 2 cc. per 10 cc. of 5 per cent gelatin); *i.e.*, the number of free carboxyl groups

[6] Van Slyke, D. D., *J. Biol. Chem.*, 1911–12, x, 49.

is doubled. Since in completely hydrolyzed gelatin the free carboxyl groups (or the amino nitrogen) is increased about twenty times, the hydrolysis due to pepsin alone corresponds to about 5 per cent of the total hydrolysis. It will be seen from the figure that this result is independent of the amount of pepsin added and also of the stage of hydrolysis at which the pepsin is added. It is not possible to say, however, whether or not this really represents the complete action of the pepsin, since the hydrolysis is still continuing slowly and if the analyses were made at weekly intervals it would be found that the values were increasing. This slow increase, however, approaches asymptotically the increase due to the acid alone, so that it is impossible to say when the action of the pepsin stops. The real end-point for pepsin digestion could be definitely fixed only by reaching the same value from both sides. For the purpose of these experiments it is permissible to take the end-point as that point at which the addition of more pepsin causes no further hydrolysis in the course of 3 or 4 days (the action of the acid alone in this time is within the limits of error of the method). In the experiment just described this value evidently corresponds to a titration of 4 cc. of 0.1 N NaOH per 10 cc. of 5 per cent gelatin.

Action of Pepsin on Gelatin Partially Hydrolyzed by Trypsin.

The results of this experiment are shown in Fig. 2. The experiment shows that the increased hydrolysis due to the pepsin becomes less the farther the hydrolysis due to the trypsin has been carried. The linkages which are split by pepsin are also evidently attacked by trypsin. At the same time, however, the trypsin is also hydrolyzing some linkages which are not attacked by pepsin. This is shown by the fact (Table I) that the addition of pepsin to a solution which has been hydrolyzed by trypsin to a titration of 7.0 cc. causes a still further increase, although, as we have seen, pepsin alone can only carry the hydrolysis to 4.0 cc. The same result was obtained by Henriques and Gjaldbäk[2] with other proteins.

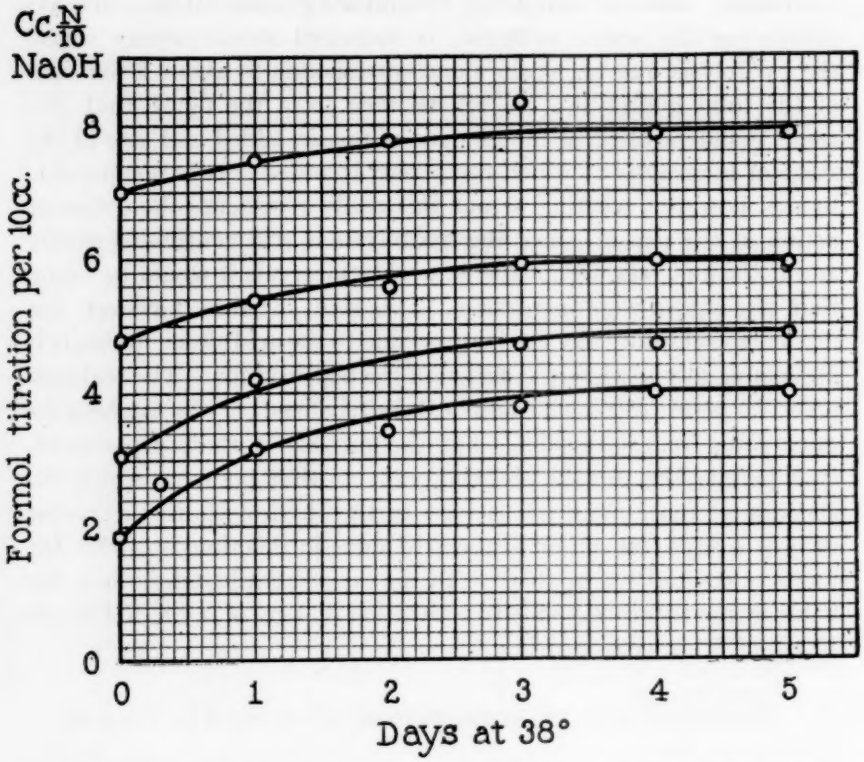

FIG. 2. Action of pepsin on gelatin previously partially hydrolyzed by trypsin. 500 cc. of 5 per cent gelatin containing 25 cc. of 1.0 M Na_2CO_3 placed at 38°C. and 5 cc. of 10 per cent dialyzed trypsin solution added (pH = 9.5). 25 cc. pipetted out at intervals and 2 cc. of 4.2 M HCl added (pH = 1.8), and the samples kept at 3° until all had been taken. 5 cc. of 5 per cent pepsin then added to each sample and the samples replaced at 38°. Formol titration was run on 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

TABLE I.

Addition of Pepsin to Gelatin Partially Hydrolyzed by Trypsin.

	Formol Titration per 10 cc. of Gelatin Solution.			
Before addition of pepsin.....	1.9	3.1	4.8	7.0
Increase in titration due to action of pepsin..	2.1	1.9	1.1	0.9

Action of Pepsin on Gelatin Partially Hydrolyzed by Alkali.

The results of this experiment are given in Fig. 3 and Table II. The results are very similar to those obtained with trypsin and pepsin. The linkages attacked by pepsin are evidently quite rapidly attacked by the alkali although some are still left after the hydrolysis has reached titration value of at least 10 cc.

Action of Pepsin on Gelatin Partially Hydrolyzed by Acid.

These experiments are summarized in Fig. 4 and Table III. They show that the linkages attacked by pepsin are among the most resistant to the action of acid since the addition of the pepsin caused the normal increase of 2 cc. even after the acid hydrolysis had proceeded to a value of over 6.0 cc. There is no evidence of a true equilibrium in the presence of pepsin since no *decrease* is noted at any time even when the acid hydrolysis has been carried far beyond the end-point reached with pepsin acting on the unhydrolyzed protein. Such a reverse or synthetic action of pepsin has occasionally been recorded (the so called plastein formation[7]) in the case of other proteins. It is apparently always connected with the formation of a precipitate. This fact makes it appear possible that the decrease in the titration value is due to the formation of some insoluble compound from substances present in the pepsin and protein solutions. This decrease has not been noted in the case of gelatin solutions and these are the ones in which no precipitate forms. The experiments described here show that the hydrolysis by pepsin follows quite a different course from that followed by acid hydrolysis. The products formed must therefore be different. It seems unlikely that the pepsin could have any synthetic action on a solution containing substances which differ from those formed by the action of pepsin itself.

Hydrolysis by Trypsin.

The extent of the hydrolysis by trypsin alone is shown in Fig. 5. The hydrolysis continues until a titration value of about 15 cc. is reached. The same uncertainty as to whether this is the real end-point evidently exists here just as in the pepsin hydrolysis.

[7] Henriques, V., and Gjaldbäk, J. K., *Z. physiol. Chem.*, 1911, lxxi, 485; 1912, lxxxi, 439.

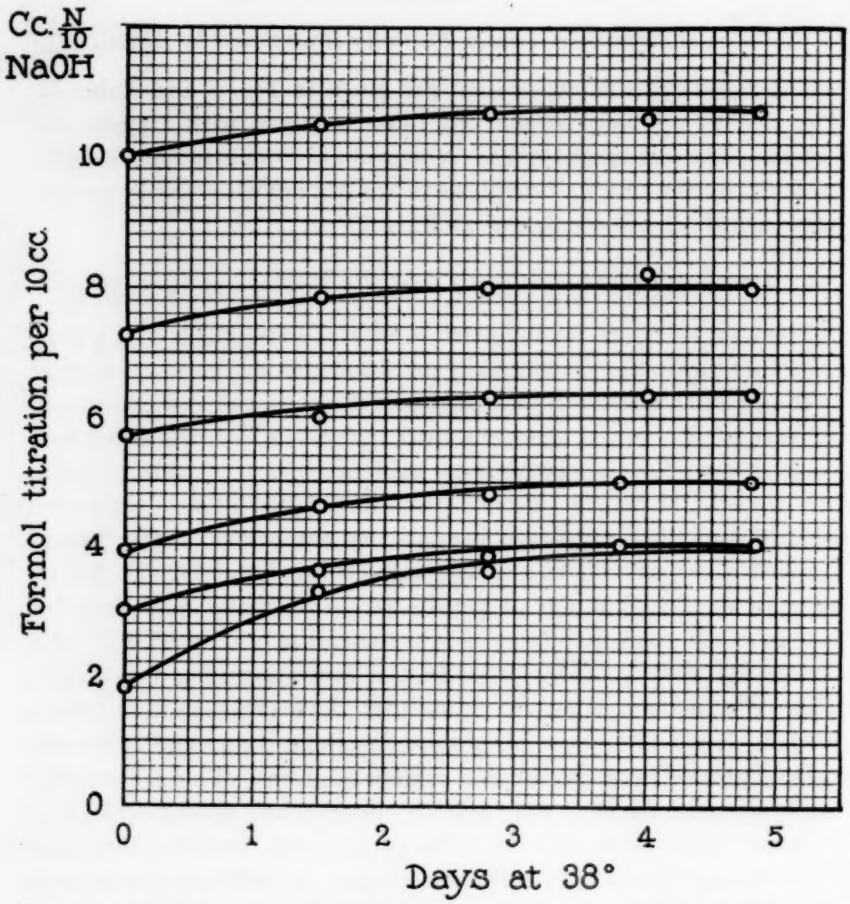

FIG. 3. Action of pepsin on gelatin partially hydrolyzed by alkali. 500 cc. of 10 per cent gelatin containing 8 cc. of 3 N NaOH kept at about 80°C. 50 cc. pipetted out at intervals and added to 50 cc. of 0.3 N HCl (pH = 2.0), these samples kept at 2° until all had been taken and 5 cc. of 5 per cent pepsin then added to each, and the samples placed at 38°. Formol titration was run on 5 or 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

TABLE II.

Addition of Pepsin to Gelatin Partially Hydrolyzed by Alkali.

	Formol Titration per 10 cc. of Solution.					
Before addition of pepsin.....	1.9	3.0	3.9	5.7	7.3	10.0
Increase due to action of pepsin.....	2.0	1.0	1.1	0.7	0.7	0.6

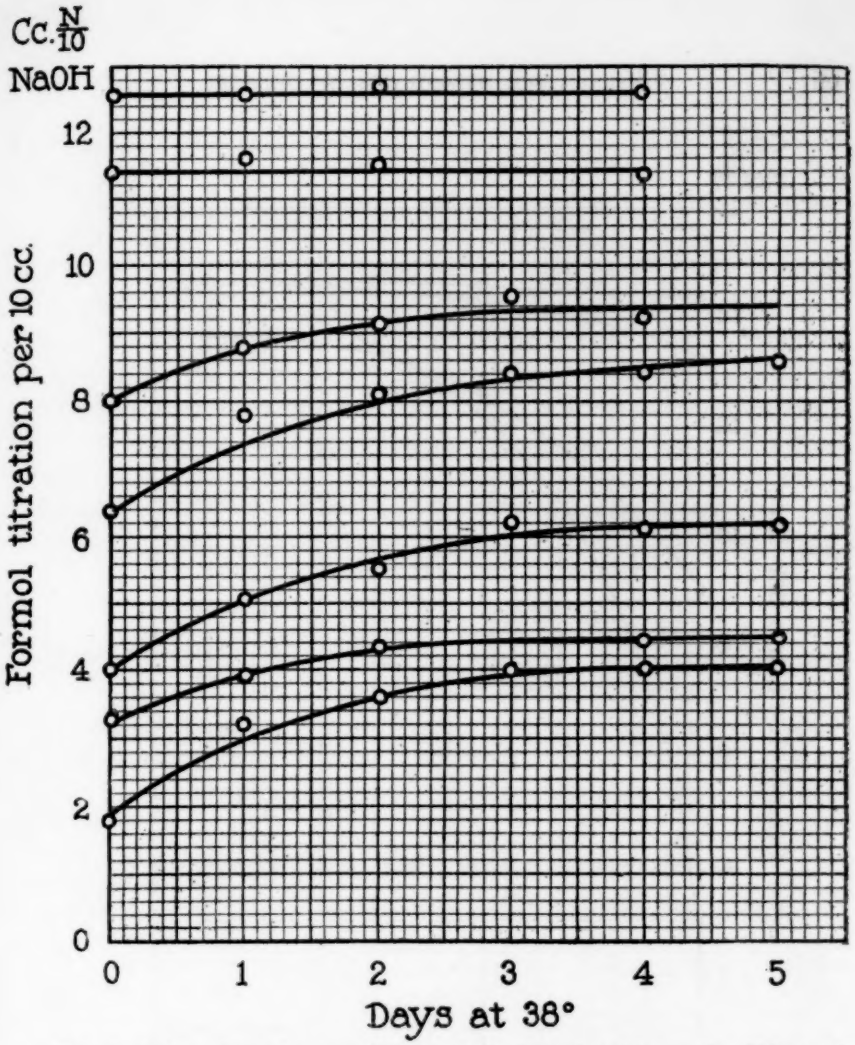

FIG. 4. Action of pepsin on gelatin partially hydrolyzed by acid. 500 cc. of 5 per cent gelatin containing 12 cc. of 4.0 M HCl (pH 1.8) kept at 38°. pH kept constant by addition of more HCl from time to time. 50 cc. samples pipetted out at intervals of 2 days and kept at 3° until all had been taken. 5 cc. of 5 per cent pepsin then added and the samples replaced at 38°. Formol titration was run on 5 or 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

TABLE III.

Addition of Pepsin to Gelatin Partially Hydrolyzed by Acid.

	Formol Titration per 10 cc. of Solution.						
Before addition of pepsin	1.9	3.3	4.0	6.4	8.0	11.4	12.6
Increase due to pepsin	2.1	(1.2)	2.0	2.0	1.0	0.0	0.0

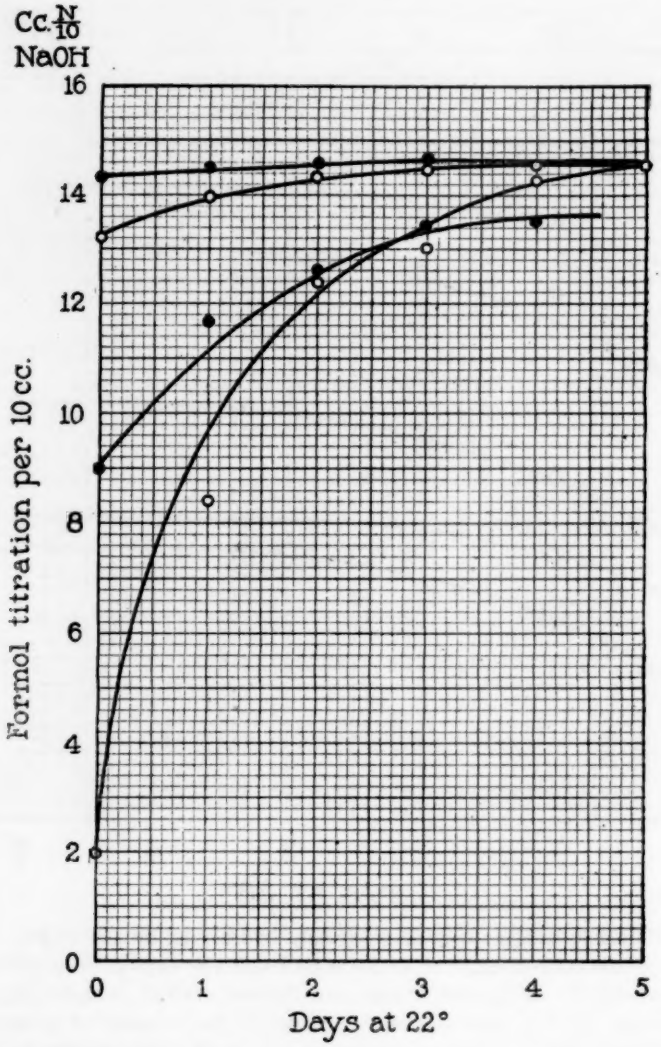

FIG. 5. Hydrolysis of gelatin by trypsin alone. 8 cc. of 10 per cent trypsin were added to 200 cc. of 5 per cent gelatin containing 10 cc. of 1.0 M Na_2CO_3, and the solution kept at 22°. 25 cc. samples pipetted out at intervals and kept at 3°C. until all had been collected. 1 cc. of 10 per cent trypsin was then added to each sample, the bottles placed at 22°, and formol titration was run on 5 cc., as shown in the figure. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

Action of Trypsin on Gelatin Partially Hydrolyzed by Pepsin.

The results of this experiment are shown in Fig. 6. The final point reached by the trypsin hydrolysis is independent of the amount of hydrolysis previously accomplished by the pepsin. The linkages hydrolyzed by pepsin are therefore evidently also hydrolyzed by trypsin so that it is immaterial whether the pepsin acts on the protein or not, as far as the final stage reached is concerned. It is possible that the *rate* of hydrolysis may be greater if the pepsin acts first since Fig. 2 showed that those linkages attacked by pepsin are among the most resistant to the action of trypsin. The experiment confirms the result shown in Fig. 2, that trypsin hydrolyzes the same linkages as does pepsin but also attacks others which are split slowly if at all by pepsin.

TABLE IV.

Addition of Trypsin to Gelatin Partially Hydrolyzed by Alkali.

	Formol Titration per 10 cc. of Solution.			
Before addition of trypsin	2.0	4.9	9.6	13.0
Increase due to trypsin	8.6	4.8	0.7	0.0

TABLE V.

Addition of Trypsin to Gelatin Partially Hydrolyzed by Acid.

	Formol Titration per 10 cc. of Gelatin.			
Before addition of trypsin	2.0	6.7	10.2	18.0
Increase due to trypsin	8.2	4.5	5.4	5.0

Action of Trypsin on Gelatin Previously Hydrolyzed by Alkali.

The results of this experiment are given in Fig. 7 and Table IV. Alkali hydrolysis evidently follows very nearly the same course as does trypsin hydrolysis since the increased hydrolysis due to the addition of trypsin becomes rapidly less and stops when the alkali hydrolysis has reached about 13 cc. This corresponds to the point reached by trypsin alone. The linkages split must therefore be the same in both cases. In this case also no evidence of a reversal of the action was observed although the experiment was carried much further than is shown in the figure.

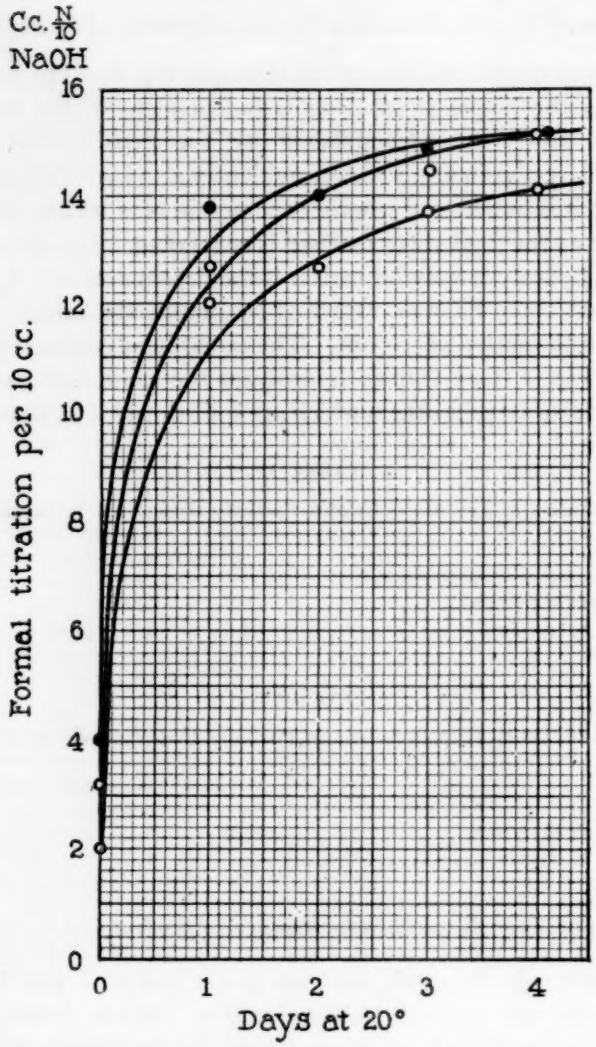

FIG. 6. Action of trypsin on gelatin partially hydrolyzed by pepsin. 500 cc. of 5 per cent gelatin solution brought to pH 2.2 with HCl, 20 cc. of 5 per cent pepsin solution added and the solution kept at 38°C. 25 cc. samples pipetted out at intervals, 5 cc. of 1.0 M Na_2CO_3 added and the samples kept at 3° until all had been collected. 1 cc. of 10 per cent dialyzed trypsin then added and the samples placed at 22°. Formol titration was run on 5 cc. samples. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

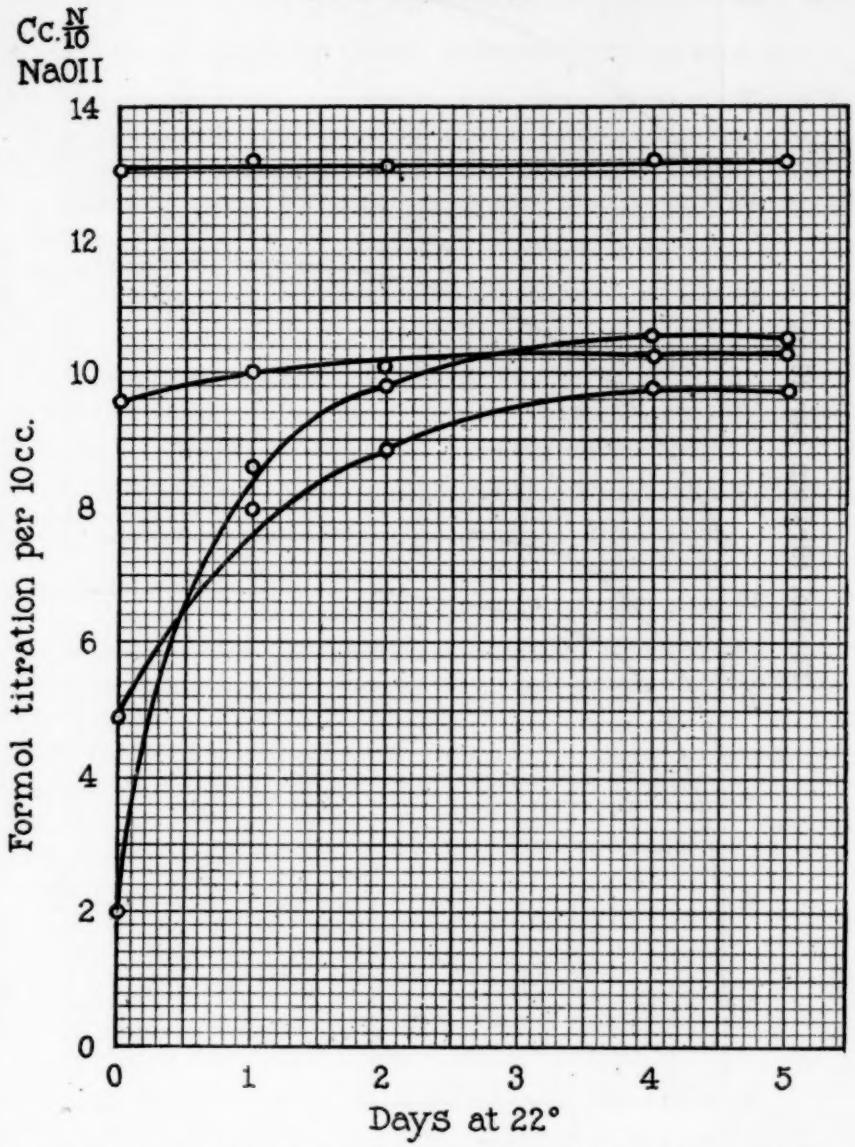

FIG. 7. Action of trypsin on gelatin partially hydrolyzed by alkali. 500 cc. of 5 per cent gelatin solution containing 50 cc. of 1.0 M Na_2CO_3 kept at 90°C. 25 cc. samples pipetted out at intervals, and kept at 3° until all had been collected. 1 cc. of 10 per cent dialyzed trypsin then added to each sample and the solutions kept at 22°. Formol titration was run on 5 cc. The figures plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

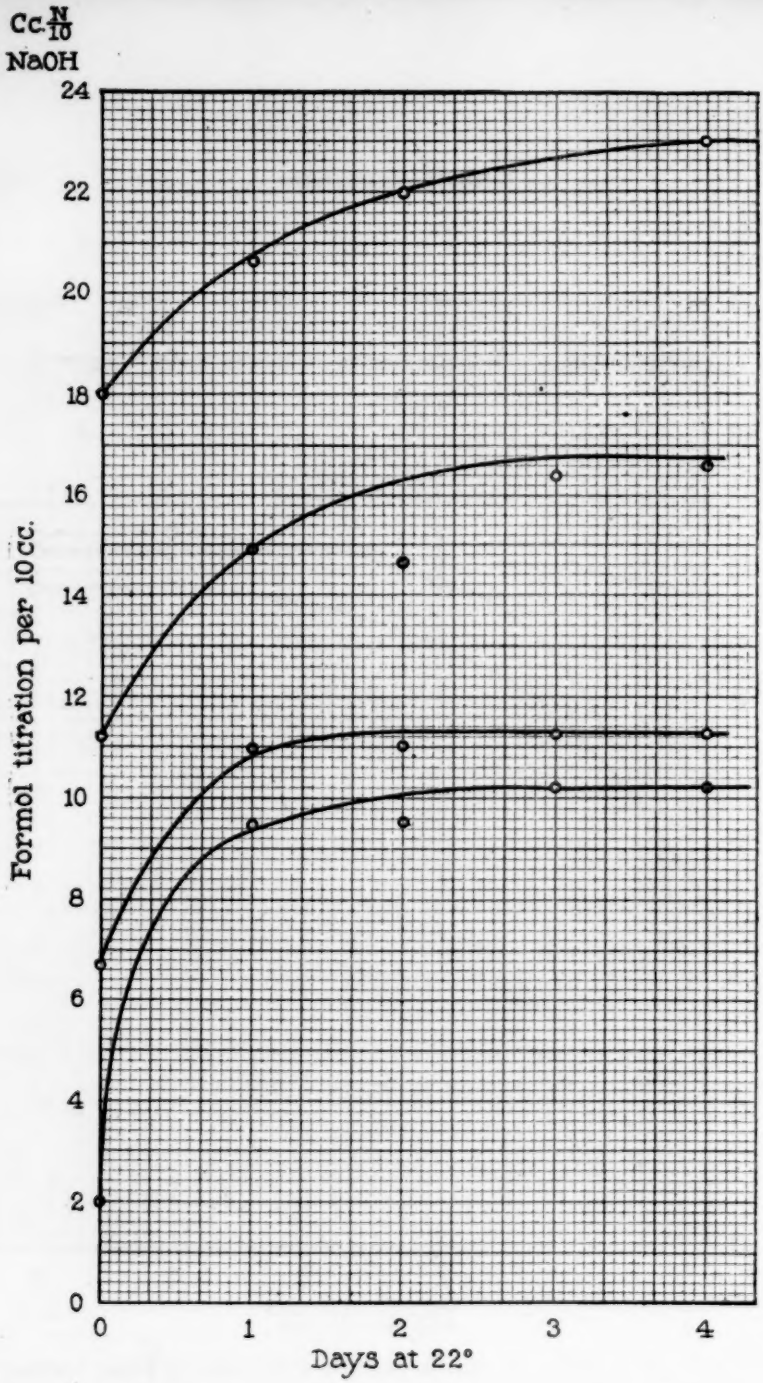

FIG. 8. Action of trypsin on gelatin partially hydrolyzed by acid. 500 cc. of 5 per cent gelatin brought to pH 1.8 with HCl and the solution kept at 90°C. 25 cc. samples pipetted out at intervals and 5 cc. of 1.0 M Na_2CO_3 added to each. Samples kept at 3° until all had been collected. They were then put at 22° and 1 cc. of 10 per cent dialyzed trypsin added to each sample. Formol titration was run on 5 cc. The figures plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

Action of Trypsin on Gelatin previously Hydrolyzed by Acid.

The results of this experiment are shown in Fig. 8, and Table V. The course of the acid hydrolysis is quite different from that due to the trypsin since the addition of trypsin to the hydrolyzed gelatin is able to cause a still further increase even after the total number of linkages already split by the acid is greater than that which could be split by trypsin alone.

SUMMARY.

A comparison has been made of the relative velocity of hydrolysis of the various peptid linkings of the gelatin molecule when hydrolyzed by acid, alkali, pepsin or trypsin. It has been found that:

1. Those linkages which are most rapidly split by pepsin or trypsin are among the more resistant to acid hydrolysis.
2. Those linkages which are hydrolyzed by pepsin are also hydrolyzed by trypsin.
3. Trypsin hydrolyzes linkages which are not attacked by pepsin.
4. Of the linkages which are hydrolyzed by both enzymes, those which are most rapidly hydrolyzed by pepsin are only slowly attacked by trypsin.
5. Those linkages which are attacked by trypsin or pepsin are among the ones first (most rapidly) hydrolyzed by alkali.

In general it may be said that the course of the early stages of hydrolysis of gelatin is similar with alkali, trypsin, or pepsin and quite different with acid.

DONNAN EQUILIBRIUM AND THE PHYSICAL PROPERTIES OF PROTEINS.

IV. VISCOSITY—*Continued.*

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1. In order to complete the demonstration that the viscosity of suspensions of powdered gelatin in water is influenced by electrolytes in a similar way as the viscosity of solutions of gelatin, it is necessary to prove that the characteristic valency effect exists also in the case of the viscosity of suspended particles of solid gelatin in water.

This proof is furnished in Fig. 1. 0.5 gm. of finely powdered gelatin (going through a sieve of mesh size 100 but not through a sieve of mesh size 120) of pH 7.0 was put into a series of beakers containing each 100 cc. of HCl of different pH and kept in the solution over night at a temperature of 20°C. Simultaneously a similar series of beakers containing each 100 cc. of H_3P0_4 and H_2S0_4 of different pH (instead of HCl) were prepared, each receiving also 0.5 gm. of powdered gelatin. After 19 hours the viscosities of all these series of suspensions were determined at 20°C. Fig. 1 gives the result, the ordinates being the values for the viscosity ratios, gelatin suspension: water, and the abscissæ are the pH of the solid gelatin particles at equilibrium. The curves show that the viscosity of suspensions of gelatin sulfate is a little less than half that of suspensions of gelatin chloride and phosphate of the same pH. The curves for the suspensions of gelatin chloride and gelatin phosphate are alike, with the exception of part of the descending branch.

Experiments on the influence of these three acids on swelling published in a previous paper[1] show that the curves for the relative volume of powdered gelatin in solutions of these three acids are similar to the

[1] **Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 253.**

viscosity curves in Fig. 1 of this paper since the relative volume of gelatin sulfate was found to be not far from one-half of that of gelatin chloride or gelatin phosphate of the same pH. This demonstration completes the proof that the viscosity of *suspensions* of powdered gelatin in water of different pH is influenced in the same way by

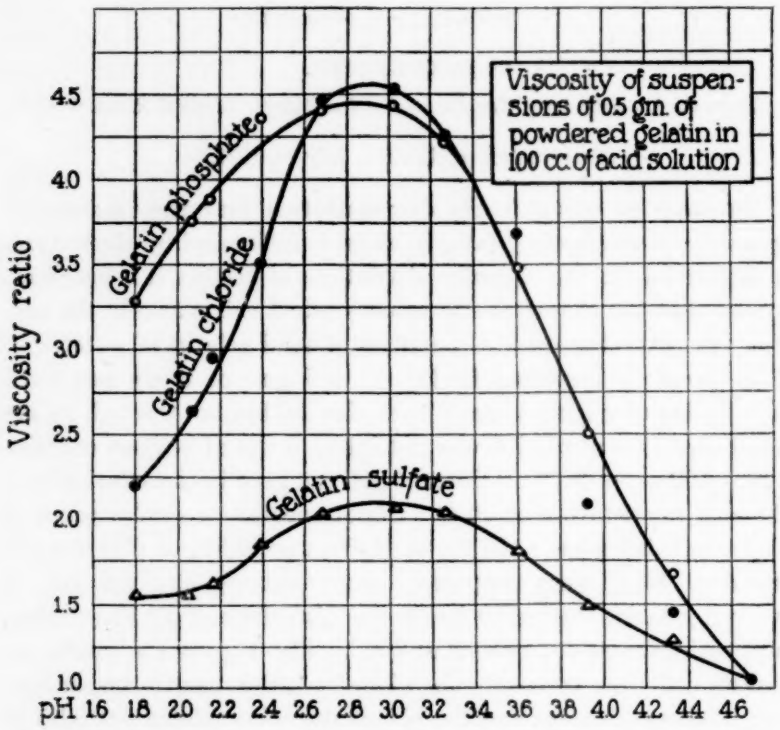

FIG. 1. Viscosity of suspensions of 0.5 gm. of powdered gelatin of grain size 100 to 120. Abscissæ are the pH, the ordinates the ratio of time of outflow of suspension: time of outflow of water. The influence of HCl and H_3PO_4 is practically identical for the same pH while H_2SO_4 depresses the viscosity of the suspensions to a little less than one-half of that for HCl.

electrolytes as is the viscosity of *solutions* of the same gelatin salts, and that this influence is due, in the case of suspensions, to the influence of the Donnan equilibrium upon the swelling of the particles.

The volume V of gelatin occupied in 100 cc. of the suspension was determined by filtering and deducting the volume of the filtrate

from the total volume of the suspension. Knowing the viscosity we can calculate Einstein's constant c according to the formula

$$\left(\frac{\eta}{\eta_0} - 1\right)\frac{100}{V} = c$$

where c should be 2.5 if V is sufficiently small.

The values in Table I show that Einstein's formula gives the correct values for viscosity when the volume, V, of the gelatin is small, since in that case c is equal, or nearly equal, to 2.5, as his formula demands.

When, however, the volume is larger, the value for c exceeds 2.5. The fact that the value for c exceeds 2.5 when the relative volume occupied by the particles in the solution is large, was found also by

TABLE I.

V	$\frac{\eta}{\eta_0}$	c
cc.		
12	1.292	2.5
17	1.480	2.8
18	1.792	4.4
20.5	2.064	5.1
20.5	2.020	4.9
20	1.855	4.2
18	1.625	3.5
16.5	1.542	3.3

Hatschek,[2] Smoluchowski,[3] and Arrhenius.[4] Hatschek replaced the value 2.5 in Einstein's formula by a larger one, namely 4.5. This, however, meets in our case with the difficulty that the value c shows a drift reaching a maximum when the volume of the gelatin particles is a maximum. This difficulty is largely avoided in Arrhenius's formula and we have to change from Einstein's formula to that of Arrhenius whenever the relative volume of the particles in solution or suspension exceeds the limits of the applicability of Einstein's formula, as we shall see in the next chapter.

[2] Hatschek, E., *Kolloid Z.*, 1913, xii, 238; 1920, xxvii, 163.

[3] Smoluchowksi, M., v., *Kolloid Z.*, 1916, xviii, 190.

[4] Arrhenius, S., *Meddelanden from K. Vetenskapsakademiens Nobelinstitut*, 1917, iii, No. 21.

The experiments on the viscosity of suspensions of powdered gelatin in water have, therefore, led to the result, first, that the influence of pH, of the valency of ions, and of the concentration of neutral salts on the viscosity of *suspensions* of finely powdered gelatin in water is similar to the influence of these three agencies on the viscosity of gelatin *solutions*; second, that the influence of electrolytes on the viscosity of the suspensions is due to the variation of the swelling (or relative volume) of the suspended particles; and third, that this latter fact explains why the Donnan equilibrium determines also the variation of viscosity of these suspensions. If it could be shown that a solution of gelatin contains also some (submicroscopic) particles of solid jelly (capable of swelling), we should understand at once why electrolytes influence the viscosity of gelatin solutions in a similar way as they influence the swelling, osmotic pressure, or the P.D. of these solutions. We have only indirect means of testing this occlusion theory of viscosity in the case of gelatin.

2. If these ideas are correct it would follow that if we melt a suspension of 0.5 gm. of powdered gelatin, in 100 cc. of water the viscosity of the 0.5 per cent solution of gelatin should be considerably lower than the viscosity of the suspension if measured at the same temperature, since in melting, part of the solid particles of the suspension should be transformed into individual molecules or ions or into particles too small to occlude water. In other words, as a consequence of the melting there should be a diminution of the relative volume occupied by the solid gelatin held originally in suspension.

This experiment was tried in the following way: 0.5 gm. of powdered gelatin was put into 100 cc. of water containing 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12.5, 15, and 20 cc. of 0.1 N HCl to bring the gelatin to different pH. The suspension was allowed to stand 1 hour at 20° to bring about the swelling of the particles and the viscosity of the suspension was measured in a straight viscometer at 20°C. The time of outflow of water through the viscometer at 20° was 48.5 seconds. The upper curve in Fig. 2 gives the ratio of viscosity of suspensions to that of water at 20°C. (When the viscosity is high, the values obtained are a little too great owing to a gravity effect which causes the solid particles to collect above the upper opening of the capillary tube during a part of the time of the experiment, thus increasing

temporarily the density of the suspension.) After the viscosity of a suspension was measured, the suspension was transformed into a solution by heating the suspension to 45° for 10 minutes; after that the solution was rapidly cooled to 20° and the viscosity of the gelatin solution was now measured with the same viscometer. The lower curve in Fig. 2 shows that the viscosity was now considerably diminished. The abscissæ are the pH of the gelatin solutions.

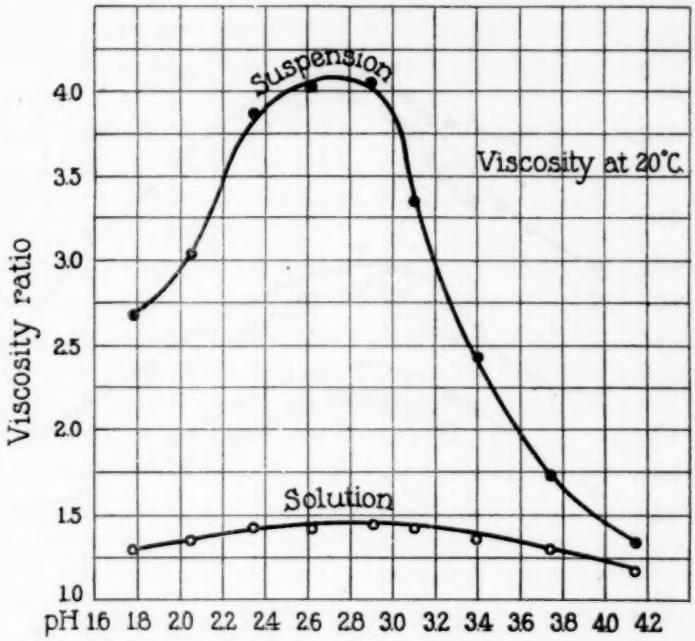

FIG. 2. Difference in the viscosity of a suspension of 0.5 gm. of powdered gelatin in 100 cc. and of the solution of the suspension in the same liquid; both viscosities were measured at 20°C.

Another test of the occlusion theory of viscosity is as follows. When we heat a 1 per cent solution of isoelectric gelatin rapidly to 45° and cool it rapidly to a lower temperature, *e.g.*, 20°C., the solution will ultimately set to a continuous gel but will steadily increase its viscosity before this stage is reached. It is natural to assume that the formation of a continuous jelly is preceded by the formation of smaller masses of jelly, which at first are submicroscopic and later

touch each other, forming a continous jelly. Hence the longer a solution of isoelectric gelatin stands at 20°C. the greater the number of submicroscopic solid pieces of jelly formed in the solution. The submicroscopic pieces of jelly surrounded by a true solution of isolated molecules of gelatin in water are compelled to regulate the amount of water they occlude by the Donnan equilibrium. Hence when we add some HCl to a 1 per cent solution of isoelectric gelatin after

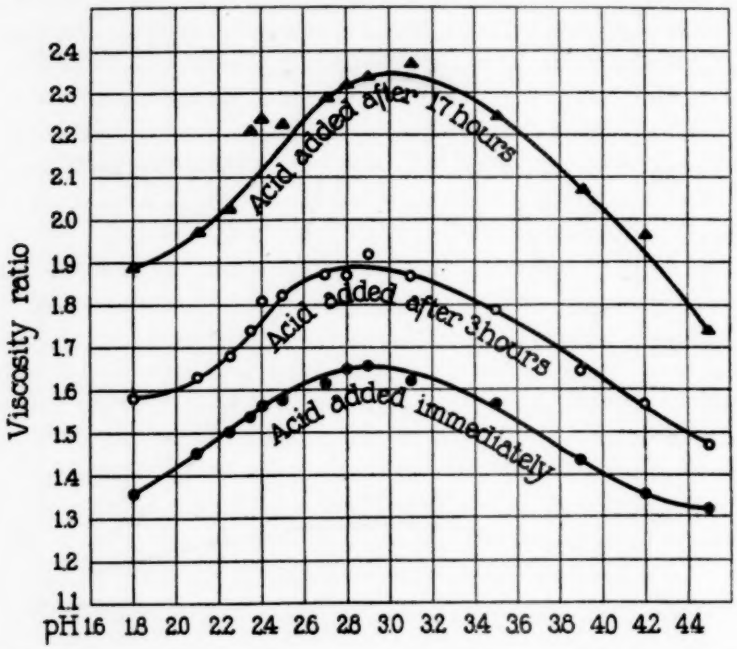

FIG. 3. Increase in viscosity when acid is added to solutions of isoelectric gelatin after they had been standing for 3 and 17 hours respectively.

the solution had been standing for some hours at 20° we should expect to find a higher viscosity than when we add the same amount of acid to the gelatin solution immediately after it had been rapidly heated to 45°C. and rapidly cooled to 20°C.

This experiment turns out as expected, as is shown in Fig. 3. When we bring newly prepared 0.5 per cent solutions of isoelectric gelatin to different pH by the addition of HCl we obtain a viscosity curve like the lowest in Fig. 3. When, however, we let the 0.5 per cent iso-

electric gelatin solution stand for 3 hours at 20°C. before adding the acid, we get a parallel curve but higher than the first one (middle curve, Fig. 3), for the reason that during the 3 hours an additional number of solid jelly particles capable of swelling has been formed. If we let the solution of isoelectric gelatin stand for 17 hours at 20°C., the curve (upper curve, Fig. 3), is still higher though practically parallel with the first curve except at the summit. It is probable that on standing the size of individual particles also increases and the greater the size the greater the viscosity, since the viscosity is chiefly but not exclusively a function of the relative volume of the particles.

These and similar experiments agree with the occlusion theory but not with the hydration theory; since there is no reason why the degree of hydration of gelatin should increase the longer isoelectric gelatin is kept at 20°C.

3. The fact that the viscosity of certain protein solutions is comparatively high, especially in the case of gelatin, has led to the suggestion that proteins might possess a different type of viscosity not present in solutions of crystalloids and that this second type of viscosity might be connected with a certain structure in the protein solution.

"Bearing in mind the possibility that protein solutions may contain a preformed molecular structure analogous to that of the jellies or coagula which they can form, we are strongly impelled towards the belief that the type of viscosity which solutions of proteins exhibit may in some manner owe its existence to this structure, and not to the type of internal friction which hinders molecular and ionic motion. Thus a netlike structure, such as a tennis net, will offer no hindrance to the passage through it of a quickly moving body which is smaller than its meshes, other than that which is due to the fact that the material which composes the net occupies a small fraction of the area which the body must traverse, but to any force which involves deformation of the structure, for instance, a force which seeks to drag it through a small tube, it will offer a very considerable resistance."[5]

[5] Robertson, T. B., The physical chemistry of proteins, New York, London, Bombay, Calcutta, and Madras, 1918, 324–325.

This assumption of a second type of viscosity seems unnecessary since it is possible to account for the viscosities of protein solutions on the basis of Einstein's law when the relative volume occupied by the protein in solution is small, and on the basis of Arrhenius's formula when the volume exceeds the limits within which Einstein's formula holds. According to our view the former is true when the protein exists in the solution exclusively or almost exclusively in the form of isolated molecules or ions or particles too small to occlude water and this seems to be the case for solutions of crystalline egg albumin, at ordinary temperature, at a pH above 1.0 and when the concentration is not excessive.

The viscosity of such protein solutions is not only of a low order of magnitude but is not influenced by electrolytes in the way as is, *e.g.*, their osmotic pressure. Where we have viscosities of a higher order of magnitude, as in the case of gelatin solutions, we notice that the viscosity is influenced by electrolytes in the same way as is the osmotic pressure of these solutions. In the case of such proteins we assume that both the high order of viscosity as well as the characteristic influence of electrolytes on the viscosity are due to the same cause; namely, the existence in the gelatin solution of a certain number of submicroscopic particles of solid jelly occluding large masses of water, the exact amount of which is regulated by the Donnan equilibrium. The occlusion of large masses of water increases the relative volume occupied by the gelatin in solution so that Einstein's formula is no longer applicable. We are dealing, however, in both cases with the same type of viscosity which is primarily a function of the relative volume occupied by the protein particles in the solution.

Measurements of the influence of concentration on the viscosity of solutions of crystalline egg albumin of pH 5.1, *i.e.*, quite near the isoelectric point, were carried out at 15°C. and showed that the viscosity is under these conditions practically a linear function of the concentration (Fig. 4). If Einstein's formula $\frac{\eta}{\eta_0} = 1 + 2.5\ \varphi$ can account for the measurements, it should be possible to calculate the volume φ of the albumin in 100 cc. of solution from

$$\varphi = \left(\frac{\eta}{\eta_0} - 1\right)\frac{100}{2.5}$$

By dividing the weight of albumin in solution by its volume, we should obtain the density of albumin which should be according to the direct determination not far from 1.36 (Arrhenius).[4] Table II shows that if we calculate the density of solutions of albumin chloride on the basis of Einstein's formula we obtain values which only vary inside the limits of accuracy of the experiments from the density deter-

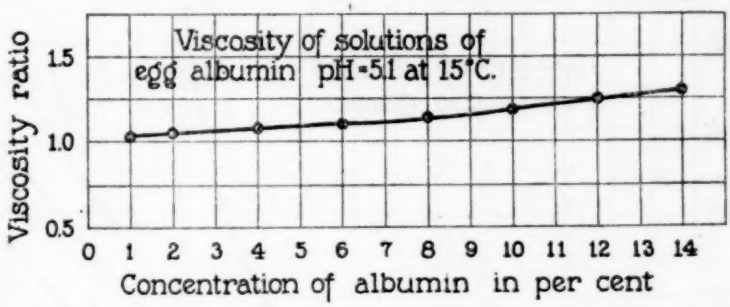
Viscosity of solutions of
egg albumin pH=5.1 at 15°C.
Viscosity ratio
1.5
1.0
0.5
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Concentration of albumin in per cent

FIG. 4. Viscosity ratio of solutions of crystalline egg albumin near the isoelectric point. Inside the concentrations used, the viscosity ratio is nearly a linear function of the concentration.

TABLE II.

Concentration of crystalline egg albumin.	$\frac{\eta}{\eta_0} - 1$	Calculated volume of albumin.	Calculated density of albumin.
per cent		*cc.*	
14	0.290	11.6	1.20
12	0.240	9.6	1.25
10	0.185	7.4	1.35
8	0.132	5.3	1.51
6	0.100	4.0	1.50
4	0.074	2.96	1.36
2	0.042	1.7	1.17

mined directly. The time of outflow of water through the viscometer was 227 seconds at 15°C.

Entirely different results should be expected in the case of solutions of gelatin if the high viscosity is due to swollen particles of solid jelly contained in the solution and not due to the existence of a network, as some authors assume. When we plot the curve for the influence of concentration on the viscosity of *solutions of isoelectric gelatin* we get

curves like those of Fig. 5, which are no longer straight lines as in the case of crystalline egg albumin but curved lines. The curvature is the smaller the higher the temperature.

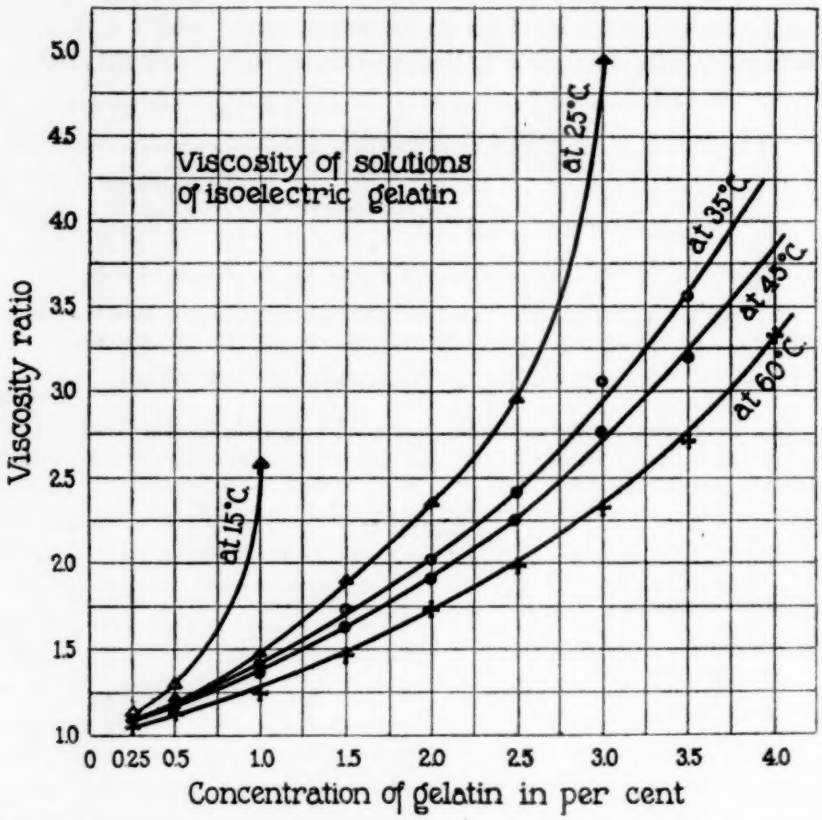

FIG. 5. Influence of concentration on the viscosity of solutions of isoelectric gelatin.

In these experiments the gelatin solution was first heated rapidly to 45° and then brought rapidly to the temperature indicated in the figure. The formula of Einstein can no longer be applied but the formula of Arrhenius gives fair agreement

$$\text{Log}\,\frac{\eta}{\eta_0} = \vartheta\varphi$$

where φ is the relative volume and ϑ is a constant. In other words, if Arrhenius's formula can account for the viscosity of these solutions the logarithm of the viscosity ratio when plotted over the concentration (Fig. 6), should give a straight line. The agreement of the value for 45° and 35° with this theory is satisfactory (considering the limits of accuracy of the measurements), (Fig. 6) the logarithms

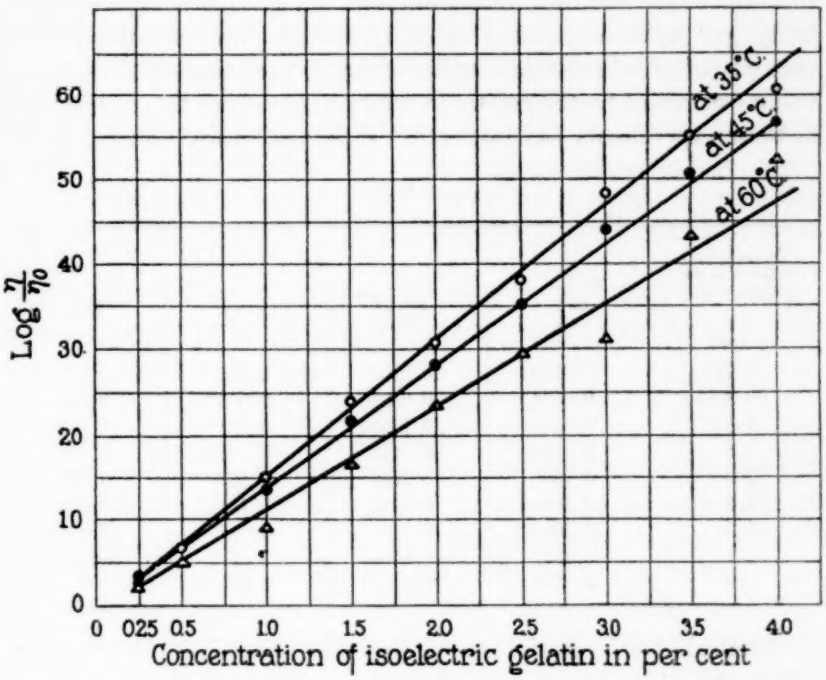

FIG. 6. Showing that the logarithms of the viscosity of solutions of isoelectric gelatin of different concentrations when plotted over the concentration as abscissæ are straight lines.

of the viscosity increasing practically in direct proportion with the concentration (*i.e.*, the relative volume) of the gelatin in the solution (Table III). At 60° the agreement is not quite so good but still recognizable. At 25°, however, it is satisfactory only at the lowest concentrations but at the higher concentrations the viscosity grows more rapidly than the concentration. The reason for this, however, is obvious since at this temperature the gelatin solution solidifies

so rapidly that the viscosity measurements were no longer possible for a concentration of 3.5 per cent gelatin solution, and for this reason the value of the viscosity of a 2 or 3 per cent solution is already too high on account of the mechanical hindrances of the flow of the solution through the viscometer owing to partial solidification.

From these experiments we may therefore draw the conclusion that since the viscosity measurements of gelatin solutions agree fairly well with Arrhenius's viscosity formula the variation of the viscosity of gelatin solutions must be due to variations in the relative volume occupied by the gelatin in solution; and since the gelatin used in these experiments was isoelectric, these variations cannot possibly

TABLE III.

Concentration of solution of isoelectric gelatin.	$\frac{\eta}{\eta_0}$			
	60°C.	45°C.	35°C.	25°C.
per cent				
0.25	0.0236	0.0306	0.0269	0.0374
0.5	0.0504	0.0682	0.0682	0.0792
1.0	0.0930	0.1350	0.1475	0.1685
1.5	0.1656	0.2135	0.2367	0.2765
2.0	0.2350	0.2796	0.3057	0.3701
2.5	0.2953	0.3512	0.3811	0.4691
3.0	0.3094	0.4409	0.4832	0.6941
3.5	0.4321	0.5051	0.5514	Solidifies
4.0	0.5214	0.5660	0.6043	

be ascribed to a hydration of gelatin ions. The only other way by which the isoelectric gelatin particles can increase their volume is through the occlusion of water by submicroscopic particles of solid jelly. Since the amount of water occluded by solid jelly is regulated by the Donnan equilibrium this will explain why the influence of electrolytes on the viscosity of a gelatin *solution* is similar to that of electrolytes on the viscosity of *suspensions* of powdered gelatin in water.

The connection between the tendency of gelatin solutions to set to a jelly and their high viscosity lies therefore in this, that the setting to a jelly is preceded by the formation of submicroscopic solid particles of gelatin capable of swelling and of occluding comparatively large

quantities of water whereby the relative volume occupied by the gelatin in the solution is enormously increased. That this interpretation is correct is proved by experiments on the viscosity of solutions of casein chloride which have no tendency to set to a jelly but which contain, side by side with isolated casein ions and molecules, small particles in suspension, occluding water and capable of swelling.

4. It has already been shown in a preceding paper that the pH influences the viscosity of casein solutions in a similar way as it influences the viscosity of gelatin solutions, and we have convinced ourselves that the addition of neutral salts has a similar depressing effect on the viscosity of casein chloride solutions as it has on the viscosity of solutions of gelatin chloride. Yet casein solutions have no tendency to set to a jelly (as far as the writer has been able to observe) and we cannot therefore attribute the influence of electrolytes on the viscosity of casein chloride solutions to a second type of viscosity. But in the case of casein chloride solutions we can prove directly that the influence of electrolytes on the viscosity has its source in the swelling of casein particles.

The material used in our experiments was a fine dry powder of nearly isoelectric casein prepared after VanSlyke and Baker.[6] Particles of equal size of grain (between mesh 100 and 120) were sifted out and 1 gm. of such powder was put into 100 cc. each of solutions of HCl of different concentration to bring the casein to varying pH.[7] A microscopic examination of the granules showed that they underwent a swelling which was a minimum at the isoelectric point, which increased with increasing hydrogen ion concentration until it reached a maximum and which then diminished again with a further increase in the hydrogen ion concentration. Hence the volume of the casein particles suspended in the HCl varied in a similar way with the pH as the volume of suspended particles of gelatin.

This swelling could also be observed when the suspension was put into 100 cc. graduates and the suspended particles were allowed to settle. The volume of the sediment was a minimum at the isoelectric

[6] Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, xxxv, 127.

[7] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 547.

point increasing with increasing hydrogen ion concentration of the solution and finally decreasing again. But the curves for swelling and for volume of sediment were only parallel at the beginning, since the swelling (which occurred at once) was followed by some of the casein going into solution, or into suspension in the supernatant milky liquid. The longer the experiment lasted the smaller the volume of the sediment became and the larger the mass which went into the supernatant solution. This is expressed in Fig. 7. The upper curve represents the volume of the sediment after 1 hour. The

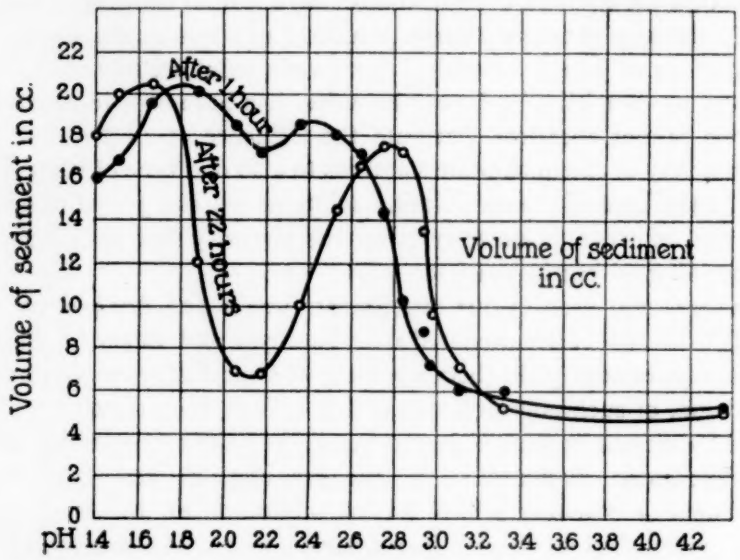

FIG. 7. Swelling and solution of casein chloride in 1 and 22 hours at 20°C.

suspension of 1 gm. of casein in 100 cc. HCl of different concentration had been kept for 1 hour at 20°, had been shaken repeatedly but not frequently and the suspension was then passed into 100 cc. graduates and allowed to settle at 20°C. After 2 hours the volume of the sediment was measured and the volumes are the ordinates of the curve marked "after 1 hour" in Fig. 7. A similar experiment was made in which the suspension of casein was kept for 22 hours at 20°C. and was allowed to settle during 6 hours also at 20°. The volumes are the ordinates of the second curve in Fig. 7 marked "after 22 hours." The abscissæ are the pH of the total solution and suspension.

The curve "after 1 hour" is clear, since it is chiefly the expression of the variation of the degree of swelling of the casein particles since not as much has gone into solution as after 22 hours. We notice that the volume occupied by the solid particles in the 1 hour curve is a minimum at the isoelectric point, that it rises steeply after pH 3.1, and it drops at 2.2, and that a second drop commences at pH 1.8. The two drops have a different cause. The drop at pH 1.8 is due to a diminution of the degree of swelling of the sediment, while the drop at 2.2 in the 1 hour curve is due to the fact that at 2.2 the solubility of casein chloride is a maximum. This conclusion is sup-

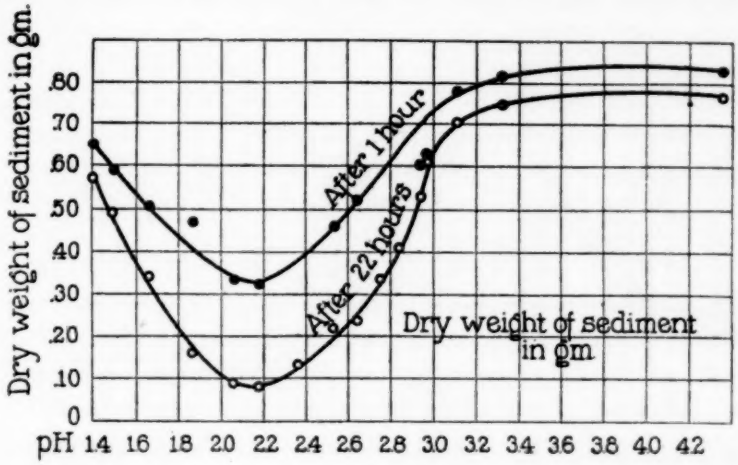

Fig. 8. Dry weight of sediment of casein chloride solutions after 1 and after 22 hours.

ported by the fact that the drop at 2.2 increases in time and is very considerable after 22 hours (Fig. 7), while otherwise the 1 hour and the 22 hour curves show only minor differences.

The proof that this interpretation in the volume curves of Fig. 7 is correct is furnished by Fig. 8 where the ordinates are the dry weights of the sediments, the volumes of which are given in Fig. 7. 1 gm. of powdered casein had when dried for 24 hours at between 90 and 100° a dry weight of 0.87 gm.

That part of the casein chloride which goes into the supernatant liquid (*i.e.*, which is not contained in the sediment) consists of two

constituents, namely, first, solid submicroscopic (and microscopic) particles in suspension which in due time would have settled, and second, isolated casein ions and molecules. The solid particles in the supernatant liquid (unless they are below the limit required to occlude water) undergo the same swelling under the influence of the Donnan equilibrium as the particles of the sediment. In addition, however, we have individual casein ions in solution (the molecules being probably insoluble since isoelectric casein is practically insoluble) but these ions cannot undergo any swelling and hence do not add materially to the volume and the viscosity. As a consequence the more solid particles of casein chloride are dissolved into isolated casein

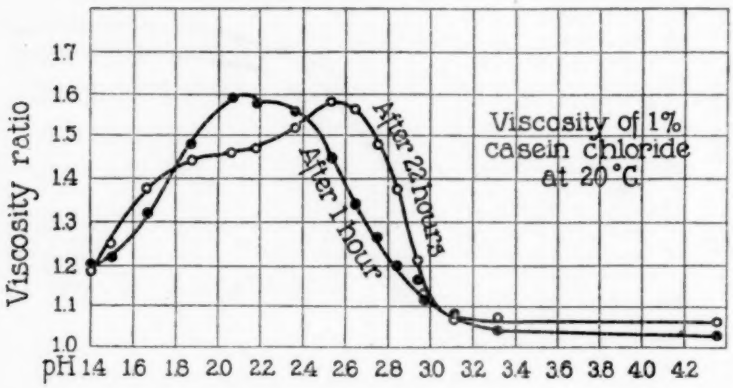

FIG. 9. Viscosity of 1 per cent casein chloride solutions after 1 and 22 hours at 20°C.

ions or particles too small to occlude water the more the relative volume occupied by the casein in the solution should be diminished and this should be accompanied by a diminution in viscosity. If our theory of the origin of the viscosity of the gelatin solutions is correct, it should be possible to prove that where the solubility of the casein chloride solution is a maximum the viscosity curve should show a drop.

The correctness of this inference is supported by the viscosity curves in Fig. 9 which represent the viscosity after 1 hour and after 22 hours. The experiments are the same as those referred to in Figs. 7 and 8. The viscosity of the total suspension and solution

was measured in a straight viscometer with a time of outflow for water of 48.4 seconds at 20°C. The curve for the viscosities after 1 hour is the expression chiefly of the swelling, since casein chloride goes only slowly into solution at 20°C. The curve is almost continuous and has its maximum in the region between pH 2.1 and 2.4, where also the swelling is a maximum. There is, however, a slight depression at pH 2.2 where the solubility of the casein is a maximum.

The curve for the viscosities after 22 hours shows, however, a distinct saddle at pH 2.2 where the solubility of casein chloride is a maximum. This agrees with the assumption that the high viscosity is due to swollen particles of casein, a certain quantity of which had been dissolved at or near pH of 2.2. This solution of the particles capable of swelling beneath that size where they no longer can occlude water must diminish the relative volume of the casein and cause a diminution of the viscosity. Below a pH of 1.8 where the solubility of the casein is considerably diminished, the 1 hour and the 22 hours viscosity curves (Fig. 9) no longer differ materially. As a consequence of the saddle the maximum of the viscosity curve after 22 hours now lies at pH 2.6.

Since the point at issue, namely the diminution of the viscosity when solid submicroscopic particles, capable of swelling, are dissolved into particles so small that they no longer can occlude water is so fundamental for the theories of viscosity and of colloidal behavior in general that it seemed necessary to look for a more striking proof than that given in the experiment quoted. For this purpose measurements were made on 1 per cent casein chloride solutions prepared from very finely powdered casein particles sifted through a 200 mesh. In order to get a more rapid dispersion of the particles the experiment was carried out at 40°C. The time of outflow of water through the viscometer at 40° was 35.5 seconds. Fig. 10 gives the results. The viscosity measurements were made at four different times; namely, first, immediately after the powdered casein was put into the HCl; then after 1, 3, and 6 hours. During this time the casein chloride solutions were kept at 40°C. The viscosity curve taken immediately after the suspensions were prepared is continuous and is the expression of the swelling which occurred in the few minutes which elapsed in the preparation of the suspensions and during

which the casein was at 40°C. The maximum swelling occurred at about pH 2.3. At this time the amount of casein dissolved into separate casein ions was negligible. The curve resembles the 1 hour curve in Fig. 9 except that the depression due to the solution of some casein chloride in Fig. 9 is lacking in Fig. 10. After $1\frac{1}{2}$ hours the second measurements of viscosity were taken, and the reader will notice from Fig. 10 that the viscosity had dropped considerably in the neighborhood of pH. 2.2, where the solubility of casein chloride is the greatest, and the maximum depression is at pH 2.1 where also the solubility is a maximum. With a further lowering of the pH the viscosity rises again. The maximal viscosity in the $1\frac{1}{2}$ hours

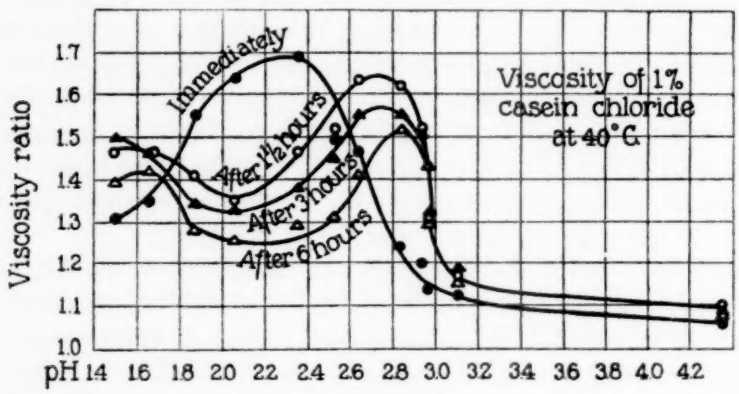

FIG. 10. Diminution of viscosity through solution of solid particles of casein chloride.

series is now at pH of about 2.7 or 2.8 where it was also in 22 hours series in Fig. 9. The later viscosity measurements, after 3 and 6 hours (Fig. 10), confirm these conclusions.

5. These experiments leave little doubt that the high viscosity of protein solutions is due to the existence of particles occluding large quantities of water the amount of which is regulated by the Donnan equilibrium while the isolated ions of proteins in solution or the particles too small to occlude water have no share in the causation of high viscosities.

The quantities of water which can be occluded in a solid jelly of gelatin are enormous. If we assume the molecular weight of gelatin

to be of the order of magnitude of about 12,000 a solid gel of 1 per cent originally isœlectric gelatin contains over 60,000 molecules of water to 1 molecule of gelatin. It is out of the question that such masses of water could be held by the secondary valency forces of the gelatin and water molecules. Swelling casein particles occlude much less water and for this reason the viscosity of casein chloride solutions never becomes as high as that of gelatin solutions, containing equal masses of protein per 100 cc. of solution.

It is well known (and we have made use of the fact earlier in this paper) that the viscosity of a gelatin solution that had been heated to 45°C. and is allowed to stand at 20° increases steadily even if the concentration is not high enough to permit the setting of the solution to a jelly. This increase in viscosity on standing seems to be caused by the gradual increase in the number of solid particles of jelly due to the collision of gelatin molecules or ions, these solid particles occluding water and thus increasing the apparent volume occupied by the gelatin in the solution. The gradual formation of solid particles of jelly from isolated molecules is most rapid at the isœlectric point and is retarded by the addition of acid; while the addition of acid increases the swelling of those particles of jelly which already exist in the solution. It is well to keep in mind that the addition of acid to a solution of isœlectric gelatin has thus two opposite effects on the viscosity of gelatin solutions.

According to Zsigmondy[8] Smoluchowski has explained (in a paper which has not been accessible to the writer) the increase in the viscosity through coagulation of a solution of aluminium oxide by the assumption of an occlusion of liquid between the particles. Smoluchowski calculates from the increase of viscosity during coagulation of aluminium oxide that the coagulating particles occupy a volume 400 to 500 times as great as that occupied by the dry material itself. This apparent increase of volume he explained through the aggregation of needleshaped particles. It would be of interest to test whether or not the volume of these particles and the viscosity of the suspension of aluminium oxide is also controlled by the Donnan equilibrium.

We are therefore led to a conception of the nature of protein solutions which is somewhat different from that current in the literature of

[8] Zsigmondy, R., *Kolloidchemie*, Leipsig, 2nd ed., 1918, 98.

colloid chemistry, which assumes that proteins form no true solutions but suspensions or emulsions. According to our view protein salts form, as a rule, true solutions consisting of isolated protein ions and molecules, which, however, may contain in addition solid fragments occluding comparatively large quantities of water. These latter particles of solid material are responsible for both the comparatively high viscosity of solutions of certain proteins, and for the influence of electrolytes on viscosity.

6. It is of interest to see whether or not Arrhenius's formula can account for the influence of electrolytes on the viscosity of casein suspensions. If this were the case, the curves representing *Log* $\frac{\eta}{\eta_0}$ should run parallel to curves representing the relative volume occupied by the casein in the solution. We get the values of Log $\frac{\eta}{\eta_0}$, from our observations of the relative viscosity which give us $\frac{\eta}{\eta_0}$, and we can calculate the volume from the measured volume of the sediment plus the calculated volume of the casein in the supernatant liquid. The latter value we obtain by deducting the dry weight of the sediment from the (known) dry weight of the whole mass of casein put into the water (1 gm. powdered casein, dry weight = 0.87 gm.), and assuming that the casein in the supernatant liquid consists exclusively of suspended particles. This is approximately correct for a 1 hour experiment at 20°. The ordinates in Fig. 11 represent the values for volume thus corrected and the values for log $\frac{\eta}{\eta_0}$ while the abscissæ are the pH of the suspensions. The two curves are almost parallel.

It should be stated that these corrected volumes of casein include a certain amount of water between the granules. We are, however, in this case not concerned with the absolute but the relative volume occupied by the casein.

When we add NaCl in different concentrations to a casein chloride solution we notice that the viscosity is diminished as it is in the case of solutions of gelatin chloride. R. F. Loeb found by microscopic observation that this diminution of viscosity was accompanied by a diminution in the degree of swelling of the individual particles of casein which ran parallel to the depression of the viscosity.

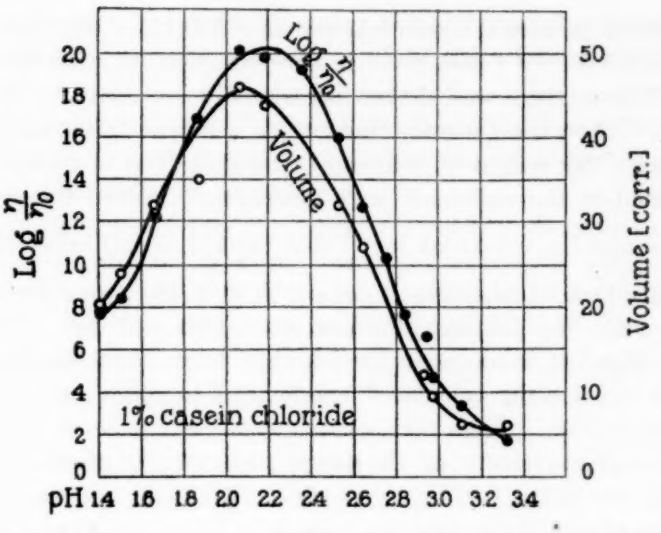

FIG. 11. Similarity of curve for log $\frac{\eta}{\eta_0}$ and of relative volume of casein chloride solutions.

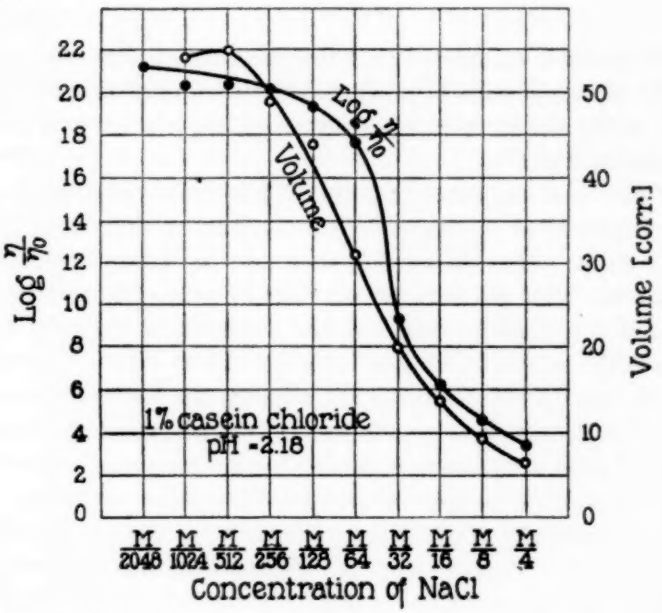

FIG. 12. Similarity of curve for log $\frac{\eta}{\eta_0}$ and of relative volume of casein chloride solutions.

1 gm. of powdered casein was put into 100 cc. of H_2O containing 12.5 cc. of 0.1 HCl and NaCl in concentrations varying from 0 to M_4. The mixture was shaken occasionally and kept for 16 hours at 20°. Then the viscosity, volume of sediment (after settling for 24 hours), dry weight of sediment (after deduction of the free NaCl contained in the sediment) were determined. When the corrected volume and the values for $\log \frac{\eta}{\eta_0}$ are plotted as ordinates over the concentration of abscissæ it is found that the two curves agree fairly well (Fig. 12) except where no or little salt was added and where therefore some casein particles had been completely dissolved. In this solution the calculated volume was too high and our curves express the fact. From these experiments we may conclude that the influence of electrolytes on the viscosity of casein solutions or suspensions is due to the swelling of particles of casein suspended in the solution of casein and that the volume of these particles is regulated by the Donnan equilibrium.

SUMMARY.

1. The proof is completed that the influence of electrolytes on the viscosity of suspensions of powdered particles of gelatin in water is similar to the influence of electrolytes on the viscosity of solutions of gelatin in water.

2. It has been suggested that the high viscosity of proteins is due to the existence of a different type of viscosity from that existing in crystalloids. It is shown that such an assumption is unnecessary and that the high viscosity of solutions of isoelectric gelatin can be accounted for quantitatively on the assumption that the relative volume of the gelatin in solution is comparatively high.

3. Since isoelectric gelatin is not ionized, the large volume cannot be due to a hydration of gelatin ions. It is suggested that this high volume of gelatin solutions is caused by the existence in the gelatin solution of submicroscopic pieces of solid gelatin occluding water, the relative quantity of which is regulated by the Donnan equilibrium. This would also explain why the influence of electrolytes on the viscosity of gelatin solutions is similar to the influence of electrolytes on the viscosity of suspensions of particles of gelatin.

4. This idea is supported by experiments on solutions and suspensions of casein chloride in which it is shown that their viscosity is chiefly due to the swelling of solid particles of casein, occluding quantities of water regulated by the Donnan equilibrium; and that the breaking up of these solid particles into smaller particles, no longer capable of swelling, diminishes the viscosity.

5. This leads to the idea that proteins form true solutions in water which in certain cases, however, contain, side by side with isolated ions and molecules, submicroscopic solid particles capable of occluding water whereby the relative volume and the viscosity of the solution is considerably increased. This accounts not only for the high order of magnitude of the viscosity of such protein solutions but also for the fact that the viscosity is influenced by electrolytes in a similar way as is the swelling of protein particles.

6. We therefore reach the conclusion that there are two sources for the viscosity of protein solutions; one due to the isolated protein ions and molecules, and the other to the submicroscopic solid particles contained in the solution. The viscosity due to the isolated molecules and ions of proteins we will call the general viscosity since it is of a similar low order of magnitude as that of crystalloids in solution; while the high viscosity due to the submicroscopic solid protein particles capable of occluding water and of swelling we will call the special viscosity of protein solutions. Under ordinary conditions of hydrogen ion concentration and temperature (and in not too high a concentration of the protein in solution) the general viscosity due to isolated ions and molecules prevails in solutions of crystalline egg albumin and in solutions of metal caseinates (where the metal is monovalent) while under the same conditions the second type of viscosity prevails in solutions of gelatin and in solutions of acid-salts of casein; and also in solutions of crystalline egg albumin at a pH below 1.0 and at higher temperatures. The special viscosity is higher in solutions of gelatin than of casein salts for the probable reason that the amount of water occluded by the submicroscopic solid gel particles in a gelatin solution is, as a rule, considerably higher than that occluded by the corresponding particles of casein.

THE RECIPROCAL RELATION BETWEEN THE OSMOTIC PRESSURE AND THE VISCOSITY OF GELATIN SOLUTIONS.

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1. If we put a suspension of 1 gm. of finely powdered gelatin in 100 cc. of HCl, so that the gelatin has a pH of 3.4, into a collodion bag and dip the latter into a pure solution of HCl (without gelatin) of pH 3.0, the liquid will practically not rise in the manometer connected with the liquid in the collodion bag (except to that slight extent which may be caused by some of the solid gelatin going into solution); in other words, we get no osmotic pressure. The reason is as follows: in this experiment the Donnan equilibrium is established between each individual granule of solid gelatin and the solution of HCl inside the bag and since the latter diffuses freely through the membrane there is in this case no difference of pH inside and outside the collodion membrane, and hence no difference of osmotic pressure on the opposite sides of the membrane (unless some of the solid gelatin goes into solution).

If we make this experiment, however, with a 1 per cent *solution* of gelatin chloride of a pH of 3.4 inside the collodion bag, while on the other side of the membrane we have a HCl solution of pH 3.0 without gelatin, we notice a rapid rise of the column of water in the manometer tube inserted into the collodion bag and the osmotic pressure of the 1 per cent solution of gelatin will be about 450 mm. H_2O or a little more when equilibrium is established.[1] This proves that the osmotic pressure of a gelatin solution cannot be due to (submicroscopic) solid particles of a jelly, but must be due to particles causing a Donnan equilibrium *across the collodion membrane*. Such particles can only be the individual gelatin ions, since even the smallest

[1] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 691.

particle of gel must cause a Donnan equilibrium to be established between the solid particles and the solution inside the bag.

If we assume that certain protein solutions contain two different constituents, namely, first, isolated ions and molecules. and second, isolated submicroscopic particles capable of occluding water, these two constituents of such solutions must play a different rôle in osmotic pressure and in viscosity. The characteristic influence of electrolytes on the osmotic pressure of protein solutions must be due to the isolated protein ions since these alone can bring about the Donnan equilibrium across the membrane; while the similar influence of electrolytes on the viscosity of protein solutions must be due to the solid submicroscopic particles since these alone are capable of swelling and of giving rise to a Donnan equilibrium between each individual particle and solution.[2] If this inference is correct, it should be possible to demonstrate the existence of a reciprocal relation between the viscosity and the osmotic pressure of those protein solutions which contain isolated protein ions as well as solid protein particles capable of occluding water; *e.g.*, gelatin or casein solutions. It is the purpose of this paper to show that this reciprocal relation between the osmotic pressure and viscosity of such protein solutions actually exists.

Fig. 1 shows that the osmotic pressure of a 1 per cent solution of originally isoelectric gelatin diminishes the more, the more we replace the dissolved gelatin by small granules of powdered gelatin. The ordinates of the upper curve represent the values of the osmotic pressure of a 1 per cent solution of originally isoelectric gelatin at different pH, the pH serving as abscissæ of the curves. The acid used was HCl, and the curve is the usual one given repeatedly in the writer's preceding publications. At the beginning of the experiment the gelatin solution was rapidly heated to a temperature of 45°C. and rapidly cooled to 20°C. and then kept at that temperature throughout the entire experiment. The pH is that of the gelatin solution at the end of the experiment.

The middle curve represents an experiment in which 0.5 gm. of the isoelectric gelatin in solution was replaced by 0.5 gm. of isoelectric powdered gelatin. The latter did not contribute to the osmotic

[2] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 73.

pressure, the observed osmotic pressure being due to the isolated ions of the 0.5. per cent gelatin in solution which determine the Donnan effect, and in addition, to the gas pressure of the isolated gelatin ions and the isolated gelatin molecules. Theoretically, of course, the

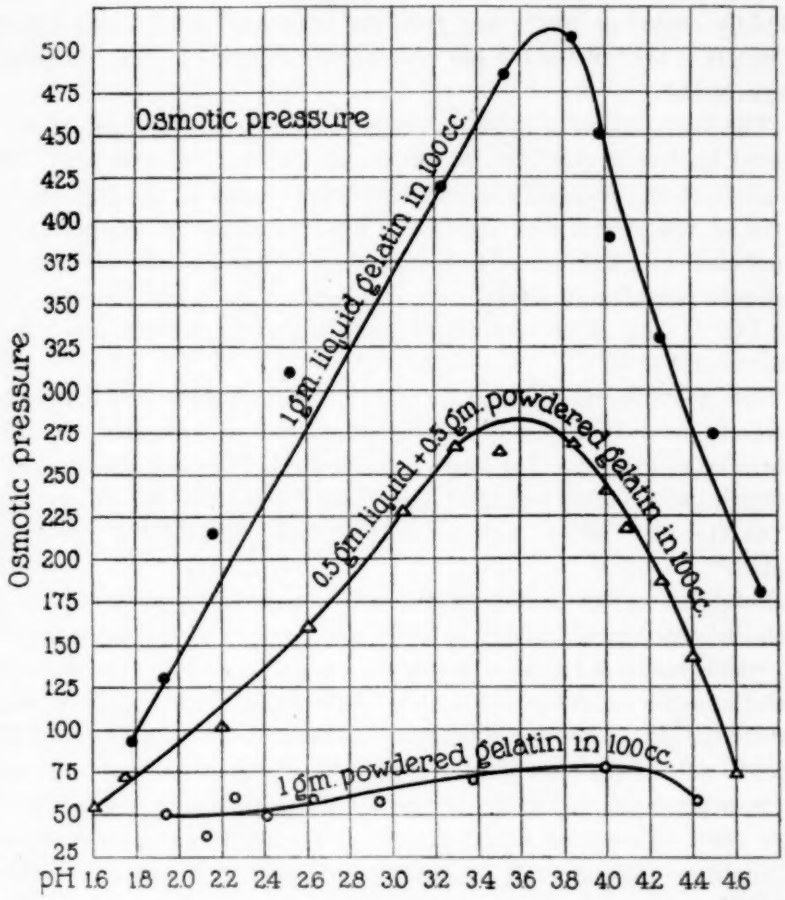

FIG. 1. A suspension of 1 gm. of a fine powder of gelatin in 100 cc. of water has practically no osmotic pressure (lowest curve), while a solution of 1 gm. of the same gelatin has a maximal osmotic pressure of over 500 mm. (uppermost curve). A mixture of 0.5 gm. of powdered and 0.5 gm. of liquid gelatin in 100 cc. water has practically the osmotic pressure of the 0.5 per cent liquid gelatin in 100 cc. of water (middle curve).

coarse particles of gelatin also participate in the osmotic pressure but this effect is negligible on account of the small number of such particles. (The gelatin particles used were of grain size slightly above one sixtieth of an inch diameter.) At the beginning of the experiment the 0.5 per cent solution of gelatin was rapidly heated to 45°C. and rapidly cooled to 20°C., and then the powdered gelatin was added. The pH is that of the 0.5 per cent gelatin solution at the end of the experiment.

The lowest curve represents the osmotic pressure of 1 gm. of powdered isoelectric particles in 100 cc. of HCl of different pH. The slight osmotic pressure observed is probably due to the fact that a little of the gelatin went gradually into solution. This apparently happened to a less extent in a repetition of this experiment and the osmotic pressures observed were still lower than in the lowest curve in Fig. 1. All these osmotic pressure experiments were made in a thermostat at 20°C.

The viscosity is affected in exactly the opposite way from the osmotic pressure if part of the dissolved gelatin is replaced by solid particles of gelatin. The more dissolved gelatin is replaced by solid particles of gelatin the higher the viscosity, a result to be expected from the experiment and conclusions published in the preceding paper.[2]

Solutions of 0.5, 0.625, 0.750, 0.875, and 1.0 gm. of isoelectric gelatin were heated quickly to 45°C. and cooled quickly to 20°C. and so much powdered gelatin of pH 7.0 was added as to bring the total gelatin in 100 cc. to 1 gm.; *i.e.*, to a 0.5 per cent solution of gelatin was added 0.5 gm. of powdered gelatin (between mesh sizes 100 and 120), and to a 0.875 per cent solution of liquid gelatin was added 0.125 gm. of powdered gelatin, while no powdered gelatin was added to the 1 per cent solution of liquid gelatin. The different mixtures were brought to different pH through the addition of different quantities of HCl and the solutions were allowed to stand for 1 hour before the viscosities were measured in order to give the powdered gelatin a chance to swell. The results of the measurements are represented in Fig. 2. The reader will see that within the range of the pH between 3.6 and 1.4 the viscosity is the greater, the more liquid gelatin is replaced by powdered gelatin. This supports the idea that the

influence of electrolytes on the viscosity of gelatin solutions is due to the influence of the electrolytes on the swelling of solid submicroscopic particles of gel in the solution.

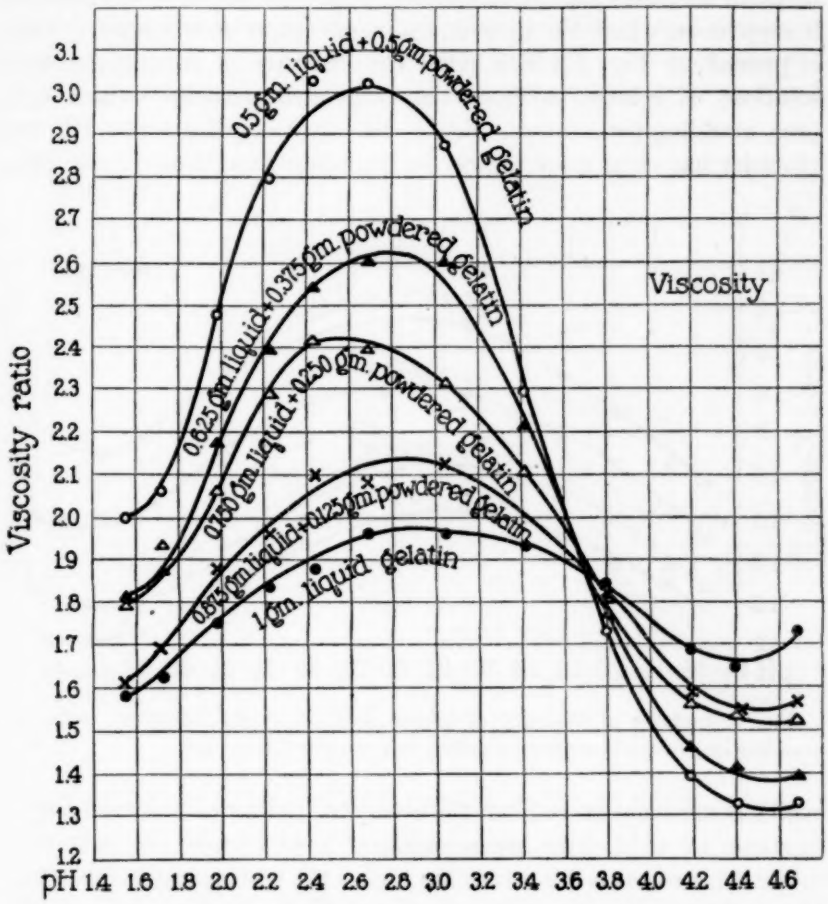
Viscosity ratio
pH
Viscosity
0.5 gm. liquid + 0.5 gm. powdered gelatin
0.625 gm. liquid + 0.375 gm. powdered gelatin
0.750 gm. liquid + 0.250 gm. powdered gelatin
0.875 gm. liquid + 0.125 gm. powdered gelatin
1 gm. liquid gelatin

FIG. 2. The influence of replacing liquid by powdered gelatin on viscosity is exactly the reverse as on osmotic pressure. The more the powdered particles replace the liquid gelatin the higher the specific viscosity.

The nature of the curves in Fig. 2 between pH 4.6 and 3.8 requires an explanation. The curves are here the lower the more liquid gelatin is replaced by solid gelatin. This is due to the fact that it was neces-

sary to let the suspensions stand for at least 1 hour to allow the particles of powdered gelatin to swell before the viscosity measurements were made. During this time the liquid gelatin at, or near, the isoelectric point increases rapidly in viscosity while this increase in viscosity is suppressed where the hydrogen ion concentration is higher. This is proved by Fig. 3 which gives the viscosity of the supernatant solutions of gelatin (without the suspended particles) which had been standing for 1 hour. Inside the range of pH 4.4 and 4.6 the viscosity had risen more rapidly on standing than at the lower pH;

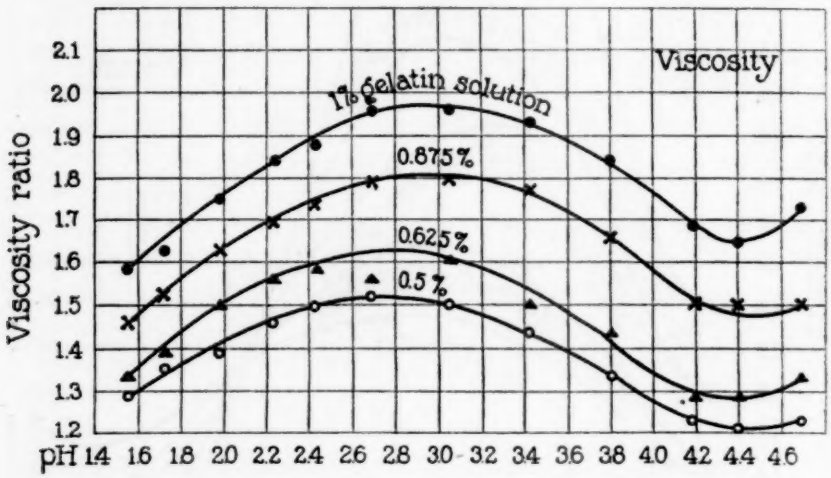

FIG. 3. Increase of viscosity of gelatin solution at the isoelectric point on standing due to the formation of submicroscopic particles of gel.

which means that at, or near, the isoelectric point new submicroscopic particles of solid jelly are constantly formed from the molecules while this process is the slower the higher the hydrogen ion concentration. While thus the addition of acid to a solution of isoelectric gelatin retards the rate of formation of new submicroscopic particles of jelly, it increases the swelling of those already present when the acid is added. On the other hand, powdered particles of isoelectric gelatin in water of pH 4.7 do not increase their volume on standing.

The fact that the addition of acid to a solution of isoelectric gelatin inhibits or retards the formation of new solid particles on standing

is strikingly illustrated by Fig. 4. Gelatin chloride solutions all containing 1 gm. of originally isoelectric gelatin were brought to various pH by adding HCl and were at the beginning of the experiment all heated rapidly to 45° and cooled at once to 20°C. Then the viscosity was measured immediately at 20°. The result is given in the lowest curve in Fig. 4. The solutions were kept at 20° for 1 hour and the viscosity measurements were repeated. The middle curve in Fig. 4

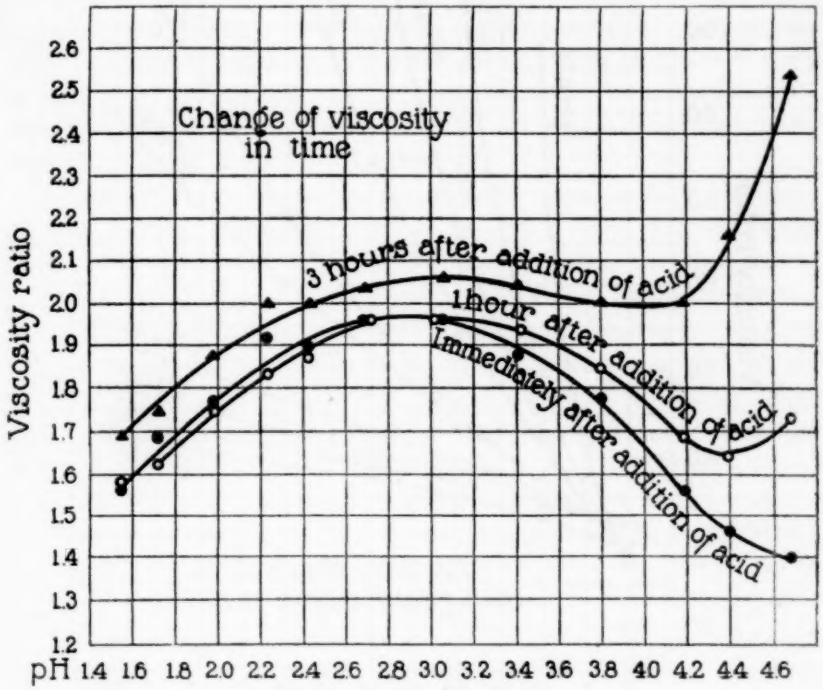

FIG. 4. See legend of Fig. 3.

shows that at this time the viscosity of the isoelectric solution had increased considerably, that of the solutions of lower pH down to 3.0 had increased the less, the lower the pH. Below pH 3.0 no increase in the viscosity had occurred. 2 hours later another viscosity measurement was made; the results are represented in the upper curve of Fig. 4. At the isoelectric point the viscosity had increased enormously, but less and less at lower pH. During all the time and also

during the viscosity measurements the temperature of the solutions was 20°C.

A more exact demonstration of the inhibiting influence of acid on the increase of viscosity of gelatin solutions on standing is given in

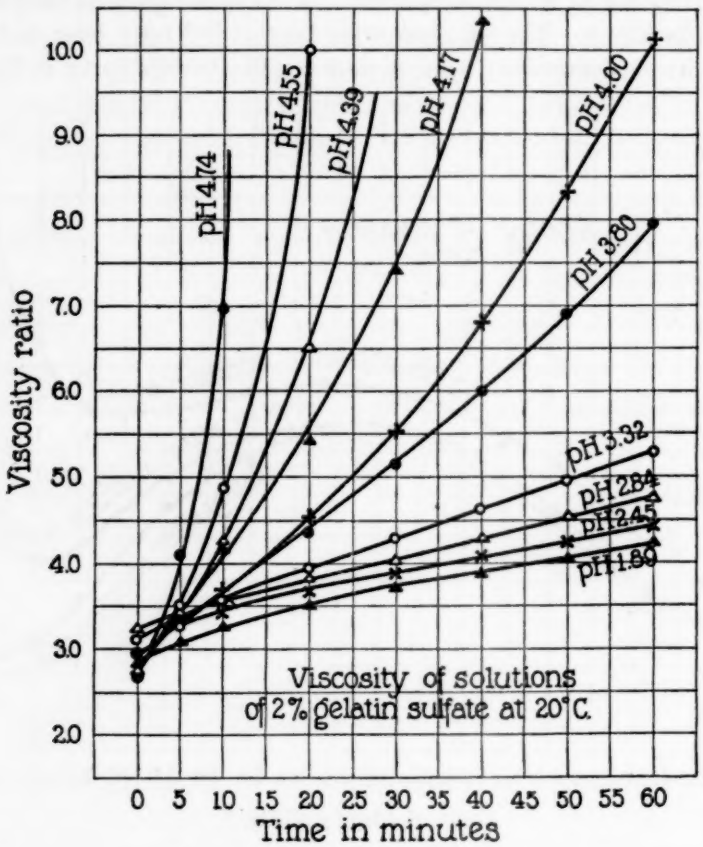

FIG. 5. Increase of viscosity of gelatin sulfate solution of different pH on standing. The increase is most rapid at the isoelectric point, thus proving that the acid retards or prevents the formation of submicroscopic solid particles of jelly on standing.

Fig. 5. Gelatin sulfate solutions containing 2 gm. of originally isoelectric gelatin in 100 cc. were prepared and heated rapidly to 45°C. and cooled rapidly to 20°C., and then kept constantly at 20°C.

Viscosity measurements were made every 5 or 10 minutes. Fig. 5 gives the result. The lower the pH the less the viscosity increases on standing.

There exist, therefore two different and to some extent antagonistic effects of acid on the viscosity of gelatin solution. One effect is due to the swelling of the submicroscopic particles of solid gelatin already present in the solution at the time the acid is added. This effect is regulated by the Donnan equilibrium and increases when (not too much) acid is added to the isoelectric gelatin solution. The second effect consists in retarding the formation of solid particles of jelly on standing. This inhibitory effect of the acid seems to increase steadily with the hydrogen ion concentration of the solution without going through a maximum at a pH above 1.8.

If we now return to the discussion of the curves in Fig. 2 we may say that the results in that part of the curves which belongs to the abscissæ of pH above 3.8 is the expression of the fact that that part of the viscosity which is due to the gelatin in solution had undergone an increase during the hour the solution had been standing at 20° after having been heated to 45°C.; and that the increase caused in the viscosity of the liquid gelatin was a maximum at the isoelectric point, being almost zero at a pH below 3.4; while the addition of acid had the opposite effect on the solid granules of gelatin, since their volume was increased according to the rules of the Donnan equilibrium.

It is necessary that we convince ourselves that a Donnan equilibrium exists when particles of solid gelatin are suspended in a solution of gelatin. That this is actually true was shown in the following way. 0.5 gm. of powdered gelatin was added to 100 cc. of a 0.5 per cent gelatin chloride solution of different pH. The different beakers containing these mixtures were kept for $3\frac{1}{2}$ hours at 20°C. The mass was then filtered through cotton wool and the pH of the filtrate (0.5 per cent gelatin solution) and of the solid gelatin granules were determined, that of the latter after they had been melted. It was found that the pH of the gelatin granules was higher than that of the solution and that the difference followed the Donnan equilibrium equation (Table I), though the result was slightly irregular owing to the fact that it is impossible to free the suspended particles of gelatin completely from the supernatant liquid. When we separate

TABLE I.

pH of gelatin in suspension....	5.12	4.60	4.49	4.18	4.07	3.73	3.45	2.93	2.68	2.34	2.09	1.86	1.77	1.53
pH of gelatin in solution.....	4.98	4.35	4.12	3.91	3.69	3.50	3.14	2.78	2.50	2.28	1.97	1.86	1.72	1.57

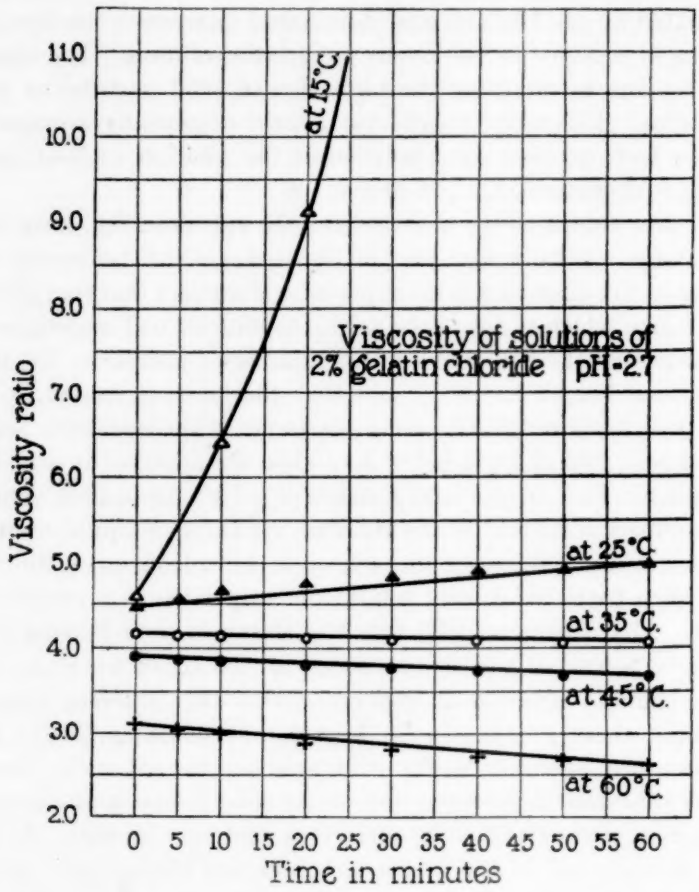

FIG. 6. Influence of temperature on the variation of viscosity of gelatin solutions on standing. Below 35°C. the viscosity of a 2 per cent gelatin chloride solution of pH 2.7 no longer increases but diminishes on standing.

a gelatin solution from water by a collodion membrane we get two equilibria, one across the membrane caused by the protein ions on one side of the membrane; and a second one inside the protein solution caused by the solid particles of jelly.

2. These experiments in which liquid gelatin was replaced by solid particles of gelatin support the idea of a reciprocal relation between the osmotic pressure and the viscosity of gelatin *solutions*. It is, however, possible to give a more direct proof. It was noticed that the viscosity of solutions of gelatin chloride does not always increase on standing but that it diminishes when the temperature exceeds a certain limit. This is shown for a 2 per cent solution of gelatin chloride of pH 2.7 in Fig. 6. The viscosity of such a solution increases on standing very rapidly at 15°C., much less rapidly at 25°C., but diminishes when kept at a temperature above 35°C., and the more rapidly the higher the temperature. This we assume to be due to the fact that at a temperature above 35° the rate of melting of submicroscopic particles of solid jelly exceeds the rate of their formation from isolated ions or molecules.

Several liters of a 0.55 per cent solution of isoelectric gelatin were kept at about 10°C. for 48 hours and at 20° for the next 24 hours. Then the stock solution was divided into two parts. The one part was divided into parts of 90 cc. each, and each part was brought to different pH by adding 10 cc. containing different quantities of HCl. In this way the concentration of originally isoelectric gelatin was, therefore, in every case 0.5 per cent. The second portion was treated in the same way except that before adding the acid the gelatin was kept for 1 hour at 45°C. This was done to melt part of the submicroscopic pieces of jelly assumed to exist in the solution and thus to increase the concentration of the isolated ions and molecules and to diminish the relative quantity of solid submicroscopic particles capable of occluding water and thereby causing the high viscosity characteristic of gelatin solutions. After this second portion of the stock solution of isoelectric gelatin had been kept for 1 hour at 45°C. it was rapidly cooled to 20°, the HCl was added in the way described for the first portion and the solutions were put into collodion bags to measure the osmotic pressure. Each collodion bag contained about 50 cc. of gelatin solution. The temperature now remained constant

at 20°C. for both sets of experiments. It was noticeable from the first that the osmotic pressure of the gelatin solution which had been kept for 1 hour at 45° and which was therefore supposed to have melted into smaller particles was higher than that of the gelatin solution not previously heated. Fig. 7 shows the result after 22 hours. The maximum osmotic pressure was, for the gelatin solution that had been previously heated, 200 mm. H_2O, while it was only 170 mm. for the other gelatin solution not previously heated to 45°C.

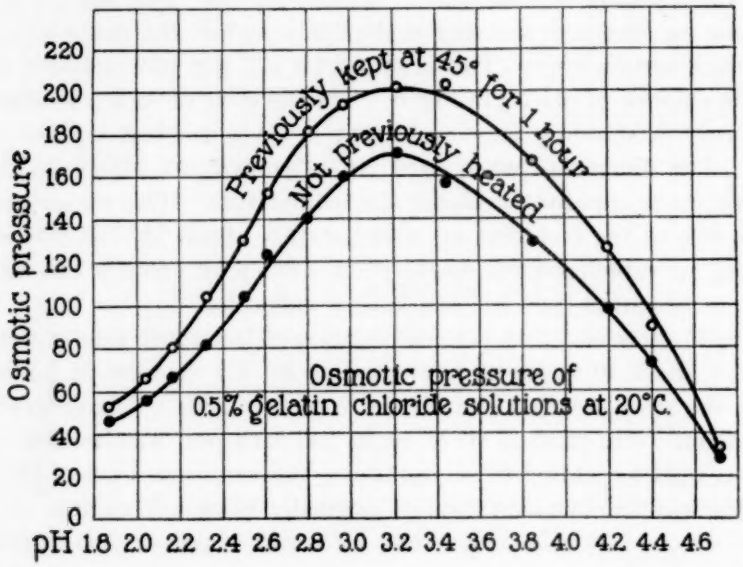

FIG. 7. Showing that the osmotic pressure of a solution of gelatin chloride which has been previously heated to 45°C. for 1 hour and then rapidly cooled to 20°C. is higher than the osmotic pressure of the same solution of gelatin chloride not previously heated.

Then the viscosities were determined at 20° and they gave the opposite result (Fig. 8), the viscosities being considerably higher in the solutions not previously heated to 45° than in the solutions previously heated. This experiment then confirms our expectation that there exists a reciprocal relation between the viscosity of protein solutions and their osmotic pressure, inasmuch as a transformation of solid submicroscopic particles of jelly into isolated protein ions

and molecules diminishes the viscosity but increases the osmotic pressure of the gelatin solution.

As far as the quantitative relations are concerned, the difference in viscosity (Fig. 8) is more striking than the difference in osmotic pressure (Fig. 7). The osmotic phenomena are a more complicated function of the change caused by the transformation of larger aggregates into simple ions, inasmuch as there exists a Donnan equilibrium not only between the gelatin solution and the outside watery solution

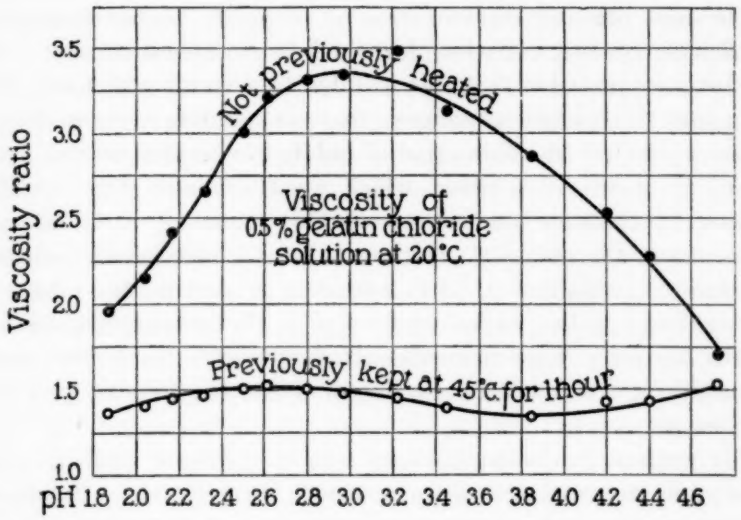

FIG. 8. Showing that the influence of previous heating on the viscosity of 0.5 per cent solutions of gelatin chloride is the reverse of the influence of the previous heating on osmotic pressure (Fig. 7).

but also between each solid particle of gelatin and the true gelatin solution surrounding it.

It was expected that when we put a 1 per cent solution of gelatin of, *e.g.*, pH 3.5, which had been kept for 1 hour at 45° and cooled to 20° into a 1 per cent solution of an identical gelatin chloride solution of pH 3.5, which had not been heated to 45° before being brought to 20°C., water would diffuse from the latter into the former solution. This experiment was carried out with a positive result.

These experiments support the idea expressed in the preceding paper that protein solutions are true solutions which may or may not contain solid particles of protein capable of swelling. In the case of gelatin solutions the formation of submicroscopic particles of solid jelly from isolated molecules or ions is a reversible process and this leads in this case to a reciprocal variation of osmotic pressure and viscosity of such solutions.

This probably explains a phenomenon which has puzzled the writer for a long time; namely, that the osmotic pressures of gelatin solutions of the same pH and concentration of originally isoelectric gelatin occasionally showed variations for which he could not account. It now becomes probable that this was due to a factor which was not taken into consideration, namely, that on standing at room temperature a gradual transformation of isolated molecules or ions into larger aggregates takes place, which must diminish the osmotic pressure but increase the viscosity. This source of variation was eliminated in the viscosity experiments in which the gelatin solution was always heated first to 45°C. and then as soon as this temperature was reached the solution was cooled to the temperature desired for the viscosity measurements. It is probable that the same uniformity of treatment is also required for the osmotic pressure experiments.

This reciprocal relation between osmotic pressure and viscosity exists probably also in the case of solutions of casein salts. Solutions of Na caseinate are less opaque than those of casein chloride (of the same concentration of originally isoelectric casein) which indicates that the Na caseinate solution contains more isolated casein ions and molecules and less submicroscopic solid particles than the solution of casein chloride.

The writer had already shown in a preceding paper that the maximal viscosity of a 1 per cent solution of casein chloride is higher than the viscosity of solutions of Na caseinate of equal concentration of originally isoelectric casein, while the osmotic pressures of solutions of the two salts show exactly the reverse relation, the maximal osmotic pressure of a 1 per cent solution of Na caseinate being almost 700 mm. H_20 while the maximal osmotic pressure of a 1 per cent solution of casein chloride is only about 200 mm.

The solutions of crystalline egg albumin seem to consist (at ordinary temperature and at not too high a concentration of albumin and of the hydrogen ions) exclusively or almost exclusively of isolated molecules or ions. Since the latter cannot diffuse through a collodion membrane they give rise to a Donnan equilibrium across the membrane and hence only the osmotic pressure of solutions of salts of crystalline egg albumin is influenced by electrolytes in the way demanded, while the viscosity shows such an influence only to a negligible degree.

3. Since in connection with viscosity we have assumed the existence of submicroscopic solid particles of jelly in gelatin solutions we must point out where our ideas agree and where they disagree with the speculations on the rôle of the degree of dispersion current in colloid chemistry. On the basis of these latter speculations it would be assumed that the osmotic pressure of a protein solution is determined directly by the concentration of the protein in solution according to van't Hoff's law. We assume the correctness of van't Hoff's law but the osmotic pressure is only in part determined by the gas pressure of the protein particles, the main source of the osmotic pressure being the unequal distribution of the crystalloidal ions on the opposite sides of the membrane due to the Donnan equilibrium. This has been overlooked by the believers in the dispersion theory. We agree with the believers in the dispersion theory that the melting down of larger aggregates of gelatin ions into isolated gelatin ions must raise the osmotic pressure but disagree in the explanation of this effect; since in our opinion the increase in the number of isolated protein ions must lead to an increase in the distribution of crystalloidal ions on the opposite sides of the membrane according to the Donnan equilibrium and this is the main reason why the osmotic pressure of a gelatin solution is increased when the solid submicroscopic particles of jelly are transformed into isolated ions.[3]

[3] If we wish to measure the true osmotic pressure of a protein solution, free from the complication of the Donnan equilibrium, we must do so at the isoelectric point of the protein. This condition was approximately fulfilled in the experiments of Sörensen (Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1915–17, xii, 262) on solutions of crystalline egg albumin, and of Hüfner and Gansser (Hüfner, G., and Gansser, E., *Arch. f. Physiol.*, 1907, 209) on solutions of hemoglobin.

On the other hand, the high viscosity of gelatin solutions, *e.g.*, that part of the viscosity which is due to the volume occupied by the solid particles of jelly, is entirely regulated by the Donnan equilibrium. The dispersion theory was not able to account for the influence of electrolytes on the viscosity and osmotic pressure of protein solutions while this influence is accounted for mathematically by the Donnan theory.

SUMMARY AND CONCLUSIONS.

1. These experiments confirm the conclusion that protein solutions are true solutions consisting of isolated ions and molecules, and that these solutions may or may not contain in addition solid submicroscopic particles capable of occluding water.

2. The typical influence of electrolytes on the osmotic pressure of protein solutions is due to the isolated protein ions since these alone are capable of causing a Donnan equilibrium across a membrane impermeable to the protein ions but permeable to most crystalloidal ions.

3. The similar influence of electrolytes on the viscosity of protein solutions is due to the submicroscopic solid protein particles capable of occluding water since the amount of water occluded by (or the amount of swelling of) these particles is regulated by the Donnan equilibrium.

4. These ideas are supported by the fact that the more the submicroscopic solid particles contained in a protein solution or suspension are transformed into isolated ions (*e.g.*, by keeping gelatin solution for 1 hour or more at 45°C.) the more the viscosity of the solution is diminished while the osmotic pressure is increased, and *vice versa*.